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LXIX. EXCESS PROTEIN AND MAMMARY SECRETION.

By GLADYS ANNIE HARTWELL.

From the Physiological Laboratory, Household and Social Science Department, King's College for Women, Kensington, London.

(*Received June 17th, 1921.*)

Introduction and Historical.

The experimental work relating to the effect of diet on mammary secretion is not extensive and what knowledge is available is of an indirect nature. Practically all the literature on this subject emphasises the fact that plenty of protein in the mother's diet is essential, but in a recently published paper [Hartwell, 1921] it was shown that excess of caseinogen in the mother's diet produced typical symptoms followed by death of the offspring, although the mother remained apparently healthy. The object of the present investigation was to find if this result were due to caseinogen specifically or to proteins in general.

Klein [1917] states that the same constituents are always present in cows' milk, but that the proportions can vary considerably. The caseinogen may be from 2 % to 5 % and the lactalbumin from 0·39 % to 0·95 %. Such variations are attributed partly to the nature of the feed and partly to external conditions, such as weather, change of milkers etc. Klein further suggests that sudden changes of feed and over-feeding should be avoided, and that milk from cows under such conditions frequently causes digestive and intestinal disturbances in infants. Larson and Putney [1917] quote an experiment performed at the Experimental Station, Wisconsin, in which some cows were fed on a heavy ration and others on a normal ration. The former did not maintain the full flow of milk during the experimental time any better than the latter, in fact "there was even a small amount in favour of the normally fed animals."

A diet of bananas, purified caseinogen and yeast or carrot-extract was fed to albino rats by Sugiura and Benedict [1918]. They found this mixture entirely suitable for the animals *except during lactation*. The mothers did well and the young flourished on the diet as soon as they could eat for themselves. No explanation of this strange result is offered, but in a later paper [1919] these observers find that adding 10 cc. of whole milk (or protein-free milk) to the above diet renders it adequate for perfect milk production of

albino rats. It seems possible in view of work quoted above [Hartwell, 1921] that this failure of the milk supply may be due to an excess of protein in the food of the mother. The proportion of protein in Sugiura and Benedict's diet based on dry constituents is 46·0 % and in experiments to be described in this paper 34·0 % protein in the mother's food is sufficient to prevent successful lactation.

Sugiura and Benedict further suggest that milk contains a new accessory substance which is needed for "suitable milk production by the mother."

Some investigations now in progress and to be published at a later date, also show that whole milk can antagonise the harmful effects produced by an excess of protein, but so far there seems no reason for postulating a new vitamin of the nature of a lactogogue.

Steenbock and Boutwell [1920] fed rats on a diet of maize, caseinogen and salts. Nine pregnancies occurred, but not one of the offspring was successfully reared. Some of the symptoms described, such as the lethargic state of the young, are similar to those to be described later, and hence it seems possible that the failure in rearing the young might be due to the mother's too great intake of protein.

The percentage of protein used by Steenbock and Boutwell is less than that of Sugiura and Benedict, but investigations have not yet gone far enough to allow of a definite statement with regard to the minimum of protein which constitutes an excess. Mitchell [1918] showed that if a large amount of protein was fed to rats, the tissues of the adult animals contained only a slight excess of amino-acids, while the tissues of the young animals contained a considerably greater proportion of amino-acids than on a normal diet.

If feeding an excess of protein to a lactating animal results in the milk having a higher proportion of protein, then the above results of Mitchell may throw some light on the fact that the mother remains apparently well, while the offspring exhibit certain typical and abnormal symptoms.

Experimental.

I. *Methods employed.*

The technique was the same as that described in a previous paper. A mixed diet of kitchen scraps and a little whole milk was given to the rats during gestation. The mothers and litters (reduced to six) were weighed daily and the protein rations started as soon as possible after the birth of the litter. Protein was given in the proportion 15·0 g. bread to 5·0 g. protein. In a few experiments a small quantity of a commercial preparation of meat extract (Jardox) was given to make the food more palatable, previous work having shown that this makes no difference to the ultimate results. The rats ate as much as they liked, the bread and protein being increased proportionately when necessary. The protein was finely ground so that the animals could not pick out and discard any of it. Water was given *ad lib.*

II. *Results.*

1. (*a*) *Diet of bread and egg albumin.* Water to drink. Fig. 1, 19. Four rats were fed on this diet. A typical curve of the weights of mother and babies is shown in Fig. 1, 19. In two of the experiments the mothers were given bread and milk for three days and the protein feeding was started on the 4th day. However, this made no difference to the final results, the babies eventually died, although for the first few days of protein feeding a better rate of growth was obtained than when the albumin and bread were given from the first day. Some of litter 19 (see curve) survived till the 17th day, another litter were all dead on the 16th day, some members of the third survived till the 20th day and three of another lived till the 26th day. These three were able to eat on the 18th day and although they were given bread and milk, they remained weak and developed bad diarrhoea.

The litters gained weight for about 12 days and up to the 10th day appeared normal, after which they became excitable. This stage of excitability lasted from two to five days and was followed by extreme weakness characterised by inability to crawl properly, a tendency to drag one hind leg and roll over towards that side. The babies were very weak and emaciated, and post mortems showed that the stomach was practically empty and the intestines had only a small amount of contents, largely mixed with gas.

Typical spasms were noticed, like those previously described when the mothers were fed with caseinogen. The babies which survived longest were sometimes able to walk though not normally. They walked on the tips of their toes (especially with the hind legs), seemed to lack co-ordinated movement, and frequently lost their balance and rolled over.

In one experiment the excitable period was very short, only one baby developed spasms and extreme weakness was evident at an early stage.

On the whole the spasms and squeaking fits were not so bad as in those litters whose mothers received caseinogen.

The mothers appeared well throughout the experiment and were able to maintain their weights, except one rat which lost slightly.

The time of onset and severity of the symptoms seem to bear no relation to the amount of food eaten by the mother.

(*b*) *Diet of bread and blood albumin.* Water to drink. Fig. 1, 15. Two rats were used in this experiment. One litter were all dead on the 16th day and the other on the 20th day. The results were identical with those described for egg albumin, except that there was a tendency in the mothers to put on weight.

2. *Diet of bread and gelatin.* Water to drink. Fig. 1, 47. Three experiments were made, and in two of them a small amount of a commercial preparation of meat extract (Jardox) was added because the animals did not eat the food very well. All three experiments, however, gave similar curves, which are represented by Fig. 1, 47. Two litters survived till the 16th day and the third

were all dead by the 11th day. Several of the babies were devoured by the mother, but others at post mortem had practically no alimentary contents. The babies showed no excitability and no spasms, but extreme weakness was evident at an early stage. Even during the first few days, the babies were

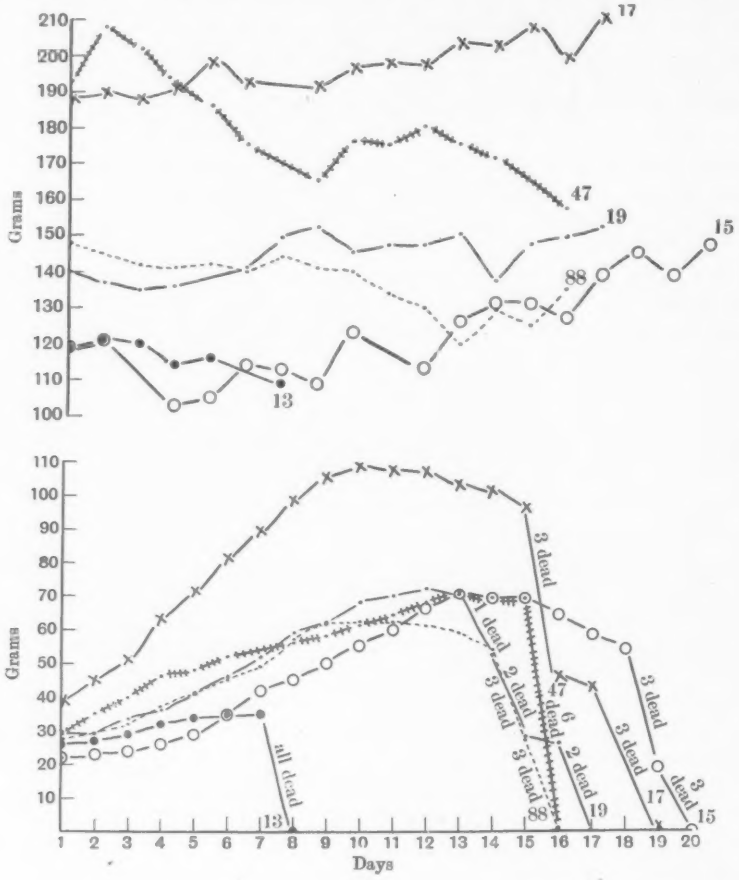

Fig. 1.

Bread and Edestin 17 Bread and Gluten 88 Bread and Gelatin 47
" " Peptone 13 " " Blood albumin 15 " " Egg albumin 19

thinner than normal ones, and compared unfavourably with those whose mothers were fed on bread alone. The tendency to drag one hind leg and roll over towards that side was very marked. The mothers lost weight considerably, one rat lost as much as 36 g. in 15 days. The significance of this is discussed in another part of the paper (p. 572).

3. *Diet of bread and gluten.* Water to drink. Fig. 1, 88. The gluten was prepared from flour by removing the starch in the usual way by kneading in muslin. When all the starch had been removed the protein was dried in an air oven and then finely ground up. Three experiments were done and the litters all died. One survived only till the 9th day, another till the 16th, and the third till the 24th day. The babies became excitable and very restless, but no actual spasms were noticed. As with gelatin extreme weakness was characteristic, and post mortems likewise showed the stomach and intestines to be practically devoid of contents.

Two of the three mothers lost weight, but the loss was not so great as with gelatin.

4. *Diet of bread and edestin.* Water to drink. Fig. 1, 17. Lactagol was used as a source of edestin. Three experiments were made and at first, in all three, a very good growth curve was obtained for the young, a greater rate of growth being shown than with any other protein (except caseinogen). The typical symptoms in the babies developed slightly later, the excitability not being shown till about the 12th day. In one experiment four of the litter survived (bad symptoms were not exhibited till the 15th day) and were successfully weaned. However they were not normal till about eight weeks old. At seven weeks they still showed the typical toe-walking (previously described) and exhibited in co-ordinated and jerky movements of their hind legs.

In the other litters the spasms were very bad, and were especially marked when the babies tried to eat. As soon as a baby took food in its mouth, the head and neck went rigid, the jaws remained open for a time, then slowly relaxed, but the rigidity returned as soon as another mouthful was taken. Post mortems showed the typical absence of contents in the alimentary canals.

In one experiment the mother was killed and the mammary glands, when cut, contained little or no milk. In all cases the mothers appeared well and either maintained or increased their weight, but the condition of the litters was as bad as that of the babies of the caseinogen-fed mothers.

5. *Diet of bread and peptone.* Water to drink. Fig. 1, 13. These experiments were not very satisfactory, because after about four days' feeding with peptone the mother had diarrhoea, possibly due to the toxic effects of the peptone. When peptone was fed to the mother immediately after the birth of the litter, the babies died within a week. In two experiments they died on the 3rd day and in another they survived a week. In this last experiment the litter gained only about 2 g. per day. The usual typical symptoms were absent, the babies merely became very weak and died. Post mortems showed absence of contents in the alimentary canals. In another experiment it was possible to continue the peptone feeding for nine days, but the mother had bread and milk for a week first, *i.e.* the litter were eight days old when the peptone feeding was started. These babies gained a small amount the first day of the peptone diet, and then lost a little each day. Two days after the peptone was given to the mother, they showed slight excitability, but no spasms. Later they

became weak, but soon ate for themselves and so survived but were weakly for some time.

6. *Diet of bread and boiled meat.* Water to drink. Fig. 2. Lean beef was used in these experiments. It was minced, extracted with distilled water in

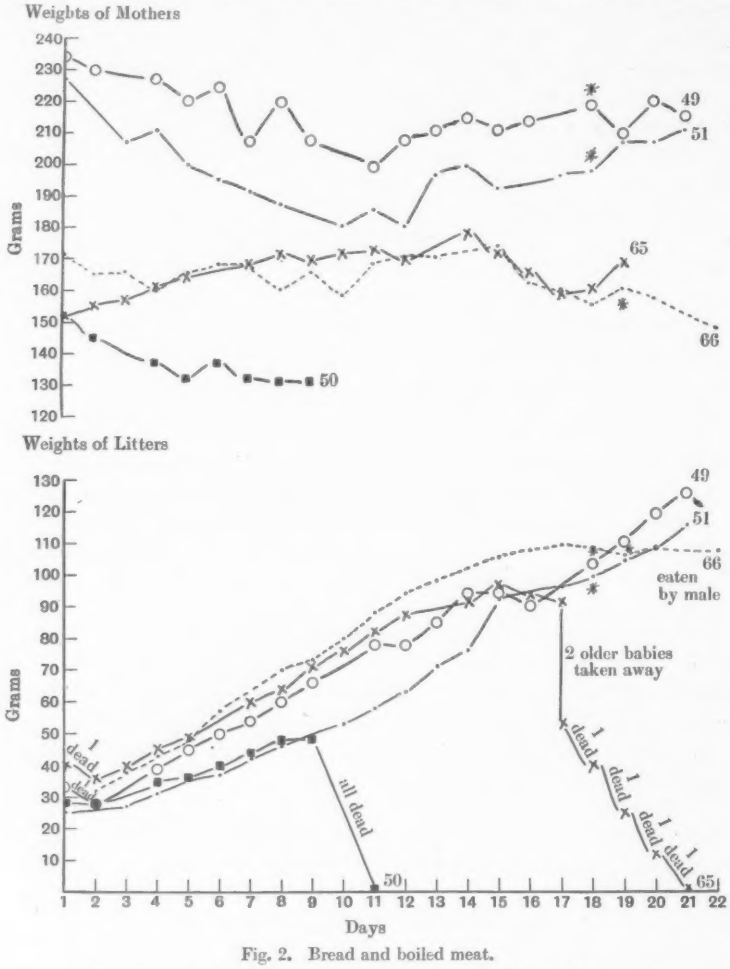

Fig. 2. Bread and boiled meat.

66	65	15 bread	5 meat		49	15 bread	15 meat	
	50	15 „	10 „		51	15 „	45 „	

* Babies eating.

a boiling water-bath for half an hour, strained and again extracted with more distilled water for three quarters of an hour. The protein was then strained through muslin and squeezed as dry as possible.

(*a*) *Experiments* 66 *and* 65. 15·0 g. *bread;* 5·0 g. *meat protein.* Rat 65 had two babies of her own and five foster babies. The former were five days old when the experiment was started. They survived and were successfully weaned, but were weakly and exhibited the typical toe-walking already described. The foster babies all developed typical symptoms, had bad spasms and squeaking fits and all died.

Litter 66 all had spasms, but survived and could eat for themselves. They never became normal[1].

(*b*) *Experiment* 50. 15·0 g. *bread;* 10·0 g. *meat protein.* The litter were all dead on the 10th day, they had no spasms, but were very weak. Post mortems proved the alimentary canals to be empty.

(*c*) *Experiment* 49. 15·0 g. *bread;* 15·0 g. *meat.* The babies were never excitable and had no spasms. About the 15th day they seemed weak and walked badly. However, they recovered, ate at the usual time and were successfully weaned.

(*d*) *Experiment* 51. 15·0 g. *bread.* 45·0 g. *meat.* The litter were absolutely normal in all respects and exhibited none of the typical symptoms at any stage.

From Fig. 2 it is easily seen that the rats 49 and 51 (whose litters did best) lost a considerable amount of weight during lactation. This is not fully understood, but in several experiments when some litters survived and others did not, the survival was coincident with a big loss in weight of the mother. Also from other experiments rats 49 and 51 are to be regarded as particularly "good milkers." Further the loss in weight of the mothers might be explained by assuming that they are mobilising an antagonistic factor from their own tissues.

7. *Diet of bread and dried meat.* Water to drink. Fig. 3. The meat was prepared as in the last series of experiments then completely dried in an air oven. The ration consisted of 15·0 g. bread and 5·0 g. dried meat. As can be seen from Fig. 3 the growth of the litters was at first fairly good, but after the 12th day there was practically no gain in weight. From this time, also, the mothers showed considerable loss in weight. The usual symptoms were absent, a few babies showed excitability and some weakness, but only to a slight degree. In all three experiments the young were not normal, but were lethargic and disinclined to move or crawl about. When taken from the cage and put on the table, they had no inclination to sniff around as would a normal rat, but remained almost motionless where they were put. In one litter three died the week after they were weaned, though fed on bread and milk. The others survived, but were weakly for some time. It is probably signi-

[1] They were all killed by the male, put in when the mother was removed. It was suggested to me by Professor Mottram that an old male is very useful for keeping with the young when they are weaned. (Technique used in Cambridge Laboratories.) As a rule it works extremely well and the male is very good to the young. He will even allow them to take food out of his mouth.

ficant that the large loss of weight of the mother coincides with the maintenance of the litters at a period when, in other experiments, the babies would have lost weight and died. As a rule when the mother is fed with protein, she either maintains or puts on weight. Whether the observed loss indicates

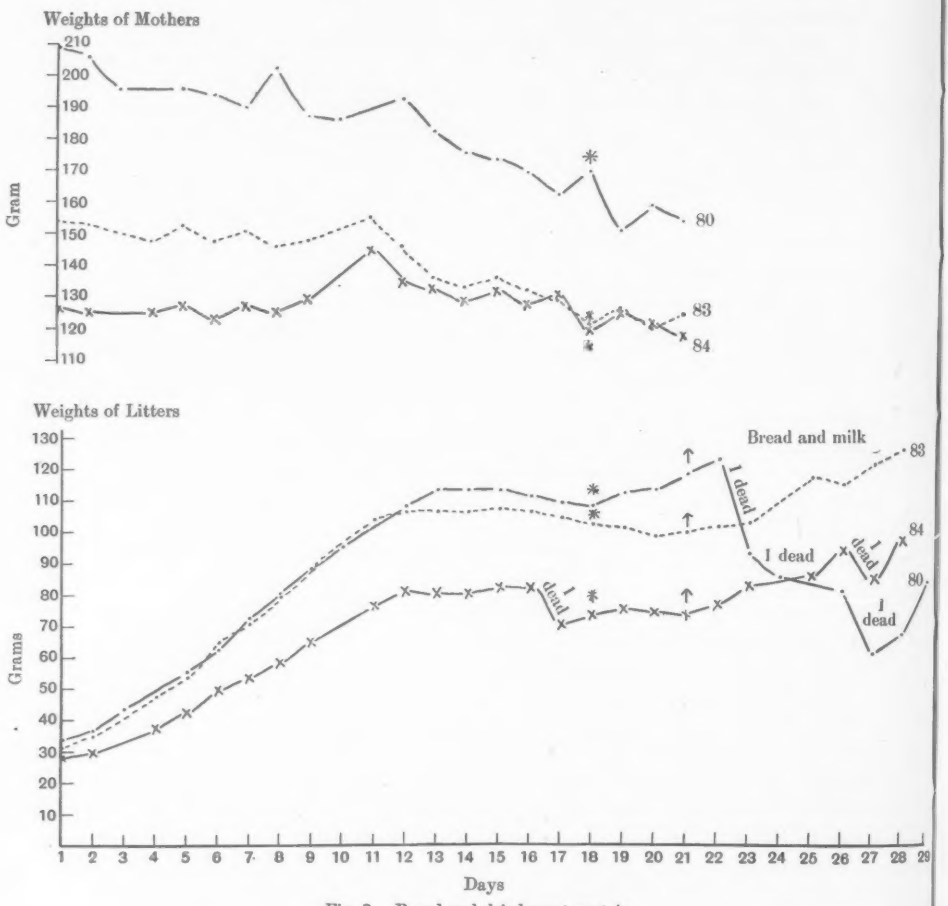

Fig. 3. Bread and dried meat protein.

* Babies eating. † Mother removed.

that the mother is supplying some essential constituent to the milk, or that she is producing some substance which inhibits the spasms, or antagonises the toxic substance responsible for this condition in the babies, remains to be proved.

8. *Diet of bread and dried fish.* Water to drink. Fig. 4. Cod was used for these experiments and the dried protein prepared in the same way as the dried meat protein. The ration consisted of 15·0 g. bread and 5·0 g. dried fish.

These results are very similar to those obtained when the mothers were fed with boiled meat. Since the individual results differ, it would perhaps be better to describe each experiment separately.

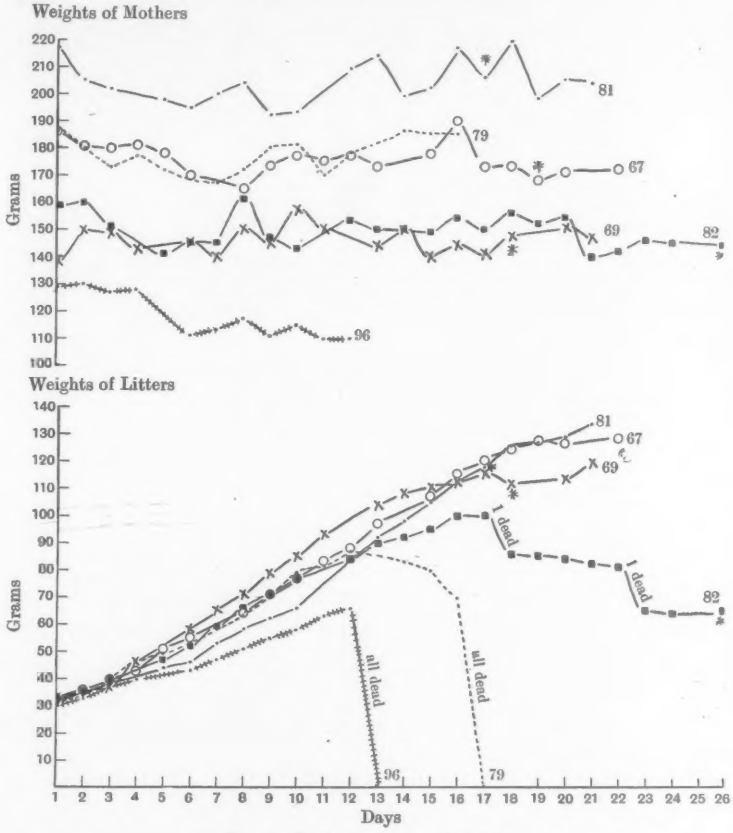

Fig. 4. Bread and dried fish protein.

* Babies eating.

Experiment 79. The growth of the litter shows a typical "protein curve" and the usual symptoms were noticed during the course of the experiment. The spasms were marked and affected all the babies at different times.

Experiment 82. The babies were normal up to the 12th day, when they were excitable, but developed no spasms.

They still gained weight until the 16th day, after which they lost small amounts each day. They were decidedly weakly, two died, and the others did not eat till the 26th day (a normal baby eats about the 18th day). Two more died on the 27th day and only one survived. This was not normal for three weeks after weaning.

Experiments 67 *and* 81. The litters appeared normal in every respect, except that they were slightly excitable and only gained small amounts towards the end of lactation.

Experiment 69. This litter resembled litters 67 and 81 except that they were more excitable and one exhibited slight spasms.

In all the experiments the mothers remained well, their weights varied somewhat but there was no great loss.

Experiment 96. 15·0 g. *bread;* 6·0 g. *dried fish.* The litter were all dead the 13th day, but the curve is by no means typical, compared with other experiments. The litter gained weight up till the 12th day, when they appeared very weak. No spasms were noticed and the usual stage of excitability was absent.

Discussion of Results.

1. *The amount of protein.* From these experiments it is clear that a lactating rat should not have a large proportion of protein in the diet. If too much protein is given, the litter rarely survive. The exact amount of protein which constitutes excess has not been determined, but it is in the neighbourhood of 33 %. It is probable that with different proteins a different proportion will constitute excess. A rat can suckle her young satisfactorily (*i.e.* the babies are normal in all respects and are successfully weaned) when fed on white bread alone [Hartwell, 1921]. Such a diet contains a very low proportion of protein, about 14 %. It is therefore possible for the rat to bring up a healthy litter when she is getting only a small amount of protein. However it is certain that a greater proportion promotes better growth, but it is obvious that such addition constitutes a danger. Where the danger line is, has to be determined by future experiments.

2. *Biological value of the proteins.* That there is some relation between the biological value of the proteins and

(i) the growth of the young,
(ii) the occurrence of spasms,
(iii) the ultimate loss of weight and death of the young, and
(iv) the mother's weight during lactation

is clear. Edestin and caseinogen were the best proteins given [see analyses by Matthews, 1916] and the young grew, showed worse spasms, and were more certain to die, while the mothers put on weight. On the most defective protein (gelatin), the young rats grew slowly for a time, became weak, showed no spasms and died. The mothers lost weight rapidly. This decrease could not be stopped by meat extract, so it is suggested that it is not due to loss

of water, but an actual tissue breakdown. There is too with other proteins a correlation, though by no means complete, between the loss of weight by the mother and the evil effects on the young. One cannot help thinking that the mammary gland cannot function properly when flooded with an excess of amino-acids and that, up to a point, the mother can antagonise that effect by mobilising something from her own tissues, perhaps vitamin, perhaps carbohydrates, perhaps salts, but improbably fats or proteins.

3. *Origin of protein.* The edestin and gelatin were commercial products, the former being used in the form of "lactagol." Hence it is possible that the results might to some extent be due to their method of preparation, or to some impurity, such as acid. Since the home-made gluten, fish and meat proteins give similar results, it is improbable that the effects are due to the "denaturing" of the proteins by commercial methods of preparation.

4. *Deficiencies in the diet.* An obvious criticism of these experiments is that a deficient diet was given, fat and vitamins being almost entirely absent. That the results are not due to the absence of these constituents is suggested by the fact referred to above, *i.e.* that a rat can bring up a healthy litter when fed with white bread alone, a diet equally deficient in fat and vitamins. But perhaps the animal may be able to tolerate a slightly greater proportion of protein when the diet is physiologically complete; the greater the amount of food metabolised the greater the need for vitamins[1].

The addition of protein makes the diet less deficient in amino-acids, except when gluten is added. Gluten with albumin forms a more balanced diet than gluten alone. None the less gluten alone is good, but gluten with other proteins, or in simple excess, is bad, therefore the bad effects on the litters must be due to the excess of protein.

5. *Variations in results caused by meat and fish proteins.* The results obtained with meat and fish proteins are obviously different from those of caseinogen, edestin, albumins, gelatin and gluten. In some experiments the litters die, exhibiting typical symptoms, exactly comparable to those seen when the mothers eat caseinogen etc. In others the litters do not suffer so badly and in a few the babies appear almost normal. Two explanations are suggested:

(i) The proteins may not be pure and consequently a smaller quantity is really being given than with pure caseinogen etc. The exact amount constituting excess has not been determined. Since the proportions used do not seem very excessive in comparison with a normal diet, it may be that the border line of excess is about the level used. If so, then the individual metabolism of the rats is a factor to be considered and what constitutes excess for one might be more easily metabolised by another. Certainly some very fine rats can tolerate more caseinogen than others.

(ii) Experiments now in progress show that the harmful effects of excess

[1] Experiments to elucidate this point are now in progress; up to the present time, no normal litters have been reared on any diet containing large amounts of protein.

protein can be antagonised by whole milk. If some specific substance in milk is responsible for this, then such a substance might be found in animal tissues, and might, therefore, be present in the meat and fish dried proteins.

Summary.

1. The observation published in an earlier paper, *i.e.* that a diet containing 40 % protein (by dried weight) fed to nursing rats, is detrimental to the young, has been confirmed and extended to commercial edestin, blood and egg albumin, gelatin and home-made gluten. Such dietaries apparently render the milk poisonous and finally check its flow.

2. Home-made fish and meat proteins do not always cause such harmful results as edestin, albumin and gelatin, but in some cases the litters die, having exhibited typical symptoms. In others the babies survive, but only a few are normal.

3. In spite of the ultimate ill effect of edestin (and caseinogen) the immediate effect of its addition to bread is to produce a maximal growth curve of the young, *i.e.* as good as when the mother's diet is bread and milk [Hartwell, 1921]. Other proteins used produce slower growth. Toxicity is not shown till the 10th or 11th day.

4. The toxicity of the mother's milk is demonstrated by the spasms etc. The cessation of the flow of milk is shown by the litter's loss of weight and the emptiness of their alimentary tracts, and this is not due to absence of suction power, because the babies are found sucking each day while they are losing weight.

5. There is some correlation between increase in the weight of the mother and danger to the offspring; if the mother puts on weight, the suckling rats suffer; if she loses weight, she may be able to spare them some of the symptoms. Gelatin, however, is an exception, because the mother loses a considerable amount of weight and yet the babies all die.

6. It is unlikely that the effect is due to an absence of vitamin, because a rat can bring up a healthy litter when she is eating white bread alone, but it is probable that it is rather due to an excess of protein in the diet. This is being worked out.

Part of the cost of these experiments was borne by the Medical Research Council to whom my best thanks are due.

I have also to acknowledge my indebtedness to Professors E. Mellanby and Mottram for criticism and encouragement.

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LXX. NOTE ON THE LIMITATIONS OF THE MODIFIED LEWIS-BENEDICT METHOD OF BLOOD SUGAR ESTIMATION.

By RUTH A. GUY.

From the Medical Department of the Royal Hospital for Sick Children and the Physiological Department of the University of Glasgow, Scotland.

(*Received June 14th, 1921.*)

During an investigation into the blood sugar in atrophic infants, in which the modified Lewis-Benedict [1915] technique was used, criticism of this method was encountered. MacLean [1919] and de Wesselow [1919] state that there are other substances in the blood which cause colour changes with picric acid. Following these suggestions, Cowie and Parsons [1920] studied the effect of various blood constituents on the Lewis-Benedict method and came to the conclusion that under pathological conditions epinephrin, acetone and diacetic acid, as well as creatinine to a less marked degree, cause a colour change in the picrate solution. They find that the picric solution is 500 times as sensitive to acetone as to glucose. Normal blood may contain 0·001 to 0·002 g. of ketones, calculated as acetone, per 100 cc. of blood. This would mean that in the normal blood, as tested by this method, 0·5 % of the reading would be due to ketones, while in diabetic bloods, in which the ketones are greatly increased, a very large percentage would be due to them. This increase in ketones added to the increase in sugar would result in observations very much higher than by other methods. This has not been the case in the hands of many competent observers.

In the present investigation decreasing amounts of acetone and ethyl aceto-acetate were added to four different samples of blood and the modified Lewis-Benedict test carried out as usual. Data are summarised in Tables I and II.

An observable colour change occurs when 0·0001 g. of acetone is added to 2 cc. of blood in 2 cc. of a 0·005 % solution. With ethyl aceto-acetate, which is probably broken down to aceto-acetic acid and ethyl alcohol, and further to acetone and carbon dioxide, during the test, no change is noted with the same amounts.

Much larger amounts of acetone and aceto-acetic acid may be added without producing any colour change if the solution is boiled before the addition of the carbonate solution.

Table I. *Acetone added to blood.*

Vol. of blood sample	Aqueous solution of acetone: Amount added	g. per 100 cc. of water	g. added	Blood sugar L.-B. test: No. 1	No. 2
2 cc. control	—	—	—	0·066	0·072
,,	2 cc.	0·005	0·0001	0·074	0·078
,,	,,	0·0025	0·00005	0·066	0·070
,,	,,	0·00125	0·000025	0·065	0·072
,,	,,	0·0005	0·00001	0·066	0·072

Table II. *Ethyl aceto-acetate added to blood.*

Vol. of blood sample	Aqueous solution of ethyl aceto-acetate: Amount added	g. per 100 cc. of water	g. added	Blood sugar L.-B. test: No. 3	No. 4
2 cc. control	—	—	—	0·070	0·079
,,	2 cc.	0·005	0·0001	0·070	0·080
,,	,,	0·0025	0·00005	0·070	0·078
,,	,,	0·00125	0·000025	0·069	0·079
,,	,,	0·0005	0·00001	0·070	0·078

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LXXI. THE COMBINATIONS OF HAEMOGLOBIN WITH OXYGEN AND CARBON MONOXIDE, AND THE EFFECTS OF ACID AND CARBON DIOXIDE.

By ARCHIBALD VIVIAN HILL.

From the Physiological Laboratory, Manchester.

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A CRUCIAL test of any theory of the mode of combination of haemoglobin with oxygen and carbon monoxide, is to attempt to explain by means of it the remarkable relations discovered by Douglas, Haldane and Haldane [1912]. It is well known that the oxygen dissociation curve of blood follows the equation,

$$y = \frac{Kx^n}{(1+Kx^n)}$$

where y is the relative degree of saturation of the haemoglobin, x the concentration of oxygen, and K a constant. This represents a tilted S-shaped curve which—whether by chance or not—certainly fits the observed points with a high degree of accuracy, and under a wide variety of conditions. This curve is affected by the presence of acid or CO_2: according to Henderson [1920] and Adair [1921] the value of $1/K$ is a linear function of the pressure of the CO_2, n remaining constant, the actual effect on the curve being to stretch it in the direction of x, without changing its shape. The first remarkable fact [Douglas, Haldane and Haldane, 1912, p. 287] is that the dissociation curve of CO is precisely similar to that of O_2, being an S-shaped curve with the same value of n, and showing qualitatively and quantitatively the same effect of CO_2. These facts can be deduced from the laws of mass action, if we assume that the reactions are governed by the equations,

$$Hb_n + nO_2 \rightleftarrows Hb_n O_{2n} \text{ (equil. const. } K),$$

$$Hb_n + nCO \rightleftarrows Hb_n (CO)_n \text{ (equil. const. } K').$$

These equations however lead immediately to a paradox. If we supposed them to apply at the same time, viz. to haemoglobin in contact with a gas containing both O_2 and CO in sufficient quantities to saturate the haemoglobin completely, and if z be the proportion of the haemoglobin combined with CO, we should find,

$$z = \frac{K'/K\,(CO/O_2)^n}{1+K'/K\,(CO/O_2)^n}$$

which, if the concentration of oxygen in the gas be kept constant, represents the CO-dissociation curve of blood in the presence of constant excess of oxygen,

and is *an S-shaped curve exactly similar in all respects to those given by* O_2 *and* CO *separately*. This however is contrary to the facts, as shown in the paper referred to (p. 278): the actual curve is a smooth rectangular hyperbola, with no trace of an inflexion in it, and completely unaffected by the presence of CO_2. Clearly there is something wrong with the simple theory. Again, from the formulae as written down above, it is obvious that decreasing the concentration of oxygen will always liberate more haemoglobin to combine with CO, and so lead to a greater CO-saturation. This again is untrue: in the paper referred to [p. 291] it is clearly shown that below a certain limit decreasing the O_2 concentration *decreases* the amount of haemoglobin combined with CO, and indeed Haldane and Lorrain Smith [1897] observed the remarkable and paradoxical fact that the administration of CO might diminish the symptoms of anoxaemia in mice subjected to low oxygen pressures.

The affinity of CO for the haemoglobin of human blood at body temperature is some 246 times that of oxygen, and if we suppose (i) that, as regards saturating the haemoglobin (without respect to the nature of the gas with which it is saturated), a mixture of a parts of O_2 and b parts of CO is precisely similar in all respects to $(a + 246b)$ parts of oxygen, and (ii) that in the resulting partly saturated and partly unsaturated mixture the saturated part is divided between O_2 Hb and CO Hb in the ratio of $a : 246b$, then all the facts established by Douglas, Haldane and Haldane can be explained. If y be the saturated fraction of the haemoglobin we then have

$$y = \frac{K(O_2 + 246\,CO)^n}{1 + K(O_2 + 246\,CO)^n}$$

of which a quantity

$$\frac{y(O_2)}{(O_2 + 246\,CO)}$$

is saturated with oxygen, and a quantity

$$\frac{y246\,(CO)}{(O_2 + 246\,CO)}$$

is saturated with CO. If $y = 1$, *i.e.* if the haemoglobin be fully saturated, it is clear that the CO-dissociation curve under constant excess O_2-concentration, is a rectangular hyperbola, and Douglas, Haldane and Haldane [1912, p. 291] showed that the paradoxical effect of diminished O_2-concentration producing a diminished CO-saturation could be explained, quantitatively and qualitatively, by the same hypothesis. The following statement of the phenomenon may make plainer what appears to be a very crucial point in the theory of haemoglobin combinations.

In Fig. 1 AB is the saturation of the haemoglobin with O_2 at O_2-pressure OB. CO gas is added to the oxygen at constant volume. The "equivalent oxygen pressure" $(O_2 + 246\,CO)$, is thus raised by $\frac{1}{3}$ (say) to OD by adding CO in pressure $BD/246$, thereby increasing the total saturation with gas to CD. Of this total saturation there are—according to the hypothesis—three parts of O_2 Hb corresponding to OB, and one part of COHb corresponding to BD, so that the oxygen saturation is now $\frac{3}{4}$ of CD, which, since CA is the steep

part of the curve, may be much larger than AB. Consequently, by adding an appropriate quantity of CO to the oxygen, the O_2-saturation has been considerably *increased*. We will consider here only the theoretical bearings of this curious and paradoxical result.

The hypothesis described above has not yet been put into physico-chemical terms. By a comparatively simple extension, however, of the physico-chemical theory which gives the S-shape to the O_2-dissociation curve, and the so-called "Hill's equation,"

$$y = \frac{Kx^n}{(1+Kx^n)},$$

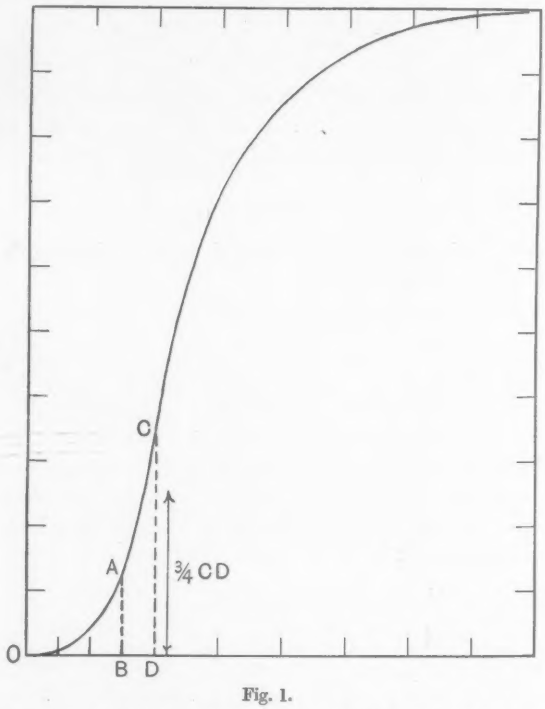

Fig. 1.

it is possible to satisfy all the relations described above, and thus to include the behaviour of O_2 and CO, separately and together, in one simple physico-chemical scheme. Let us assume that haemoglobin is a slightly dissociated compound of the iron-containing and the protein molecules, according to the equation,

$$\mathrm{Hb} \rightleftarrows \mathrm{A} + \mathrm{B},$$

where A is the protein and B is the iron-containing part. This assumption is not new, since it was a central one in Bohr's [1903] theory of the combinations of haemoglobin.

Let us assume further that in the presence of salts the osmotic pressure of the complex Hb, and of the simple protein A, is reduced to $1/n$ of its value calculated on the assumption that one molecule of haemoglobin contains one atom of iron. This assumption, as will be shown in a forthcoming paper, can be verified experimentally. It is not necessary to assume that this reduction of osmotic pressure is due to aggregation of the molecules, as was previously done [Hill, 1910]. Any cause whatever which reduces the osmotic pressure will give an S-shaped dissociation curve, as can be shown by rigid thermodynamical reasoning. The iron-containing molecule B, being small, we should expect to be unaffected in this respect, so that in the presence of salts we may assume that the above equation runs,

$$Hb_n \rightleftarrows A_n + nB. \qquad \text{.....(I)}$$

Let us now assume that the combination of haemoglobin with a gas X (either O_2 or CO) takes place only between molecules in the dissociated form, *i.e.* not between Hb and the gas, but between free B and the gas, according to the scheme,

$$B + X \rightleftarrows BX, \qquad \text{.....(II)}$$

the gas-saturated iron-containing molecule then recombining with the protein molecule according to the scheme,

$$nBX + A_n \rightleftarrows (HbX)_n. \qquad \text{.....(III)}$$

There would seem to be some evidence for this. Oxyhaemoglobin is known to be a stronger acid than reduced haemoglobin, which suggests that in reduced haemoglobin the protein molecule and the iron-containing molecule are combined by two separate linkages, while in oxyhaemoglobin the two molecules are combined by only one linkage, the spare linkage of the protein requiring sodium or some other positive ion and the spare linkage of the iron-containing molecule requiring a gas, for its saturation. Thus the combinations would appear to run as follows:

$$(A{=}B) \rightleftarrows (A{=}) + (B{=})$$
$$(=B) + X \rightleftarrows (-BX)$$
$$(A{=}) + (-BX) \rightleftarrows (-A-BX)$$
$$(-A-BX) + Na \rightleftarrows NaA-BX.$$

Now if the process of combination of haemoglobin with a gas were as suggested, the recombination of the protein with the gas-saturated iron-containing parts might be expected to take place quite independently of the nature of the gas with which the latter was combined. In other words, in equation (III) the equilibrium constant is independent of the nature of the gas X, and is concerned only with the fact that one of the affinities of B has been satisfied. In equations (I) and (III), assuming the degree of dissociation of haemoglobin to be very small, if the proportion of saturated haemoglobin $(HbX)_n$ be

called y, and the proportion of unsaturated haemoglobin Hb_n be called $(1 - y)$, we have, if K_1 and K_3 be the constants of reactions (I) and (III),

$$y = K_3(A_n)\,(BX)^n, \qquad \text{.....(IV)}$$

$$(1 - y) = K_1(A_n)\,(B)^n. \qquad \text{.....(V)}$$

Assume first that the gas X is O_2 only. Then equation (II) gives

$$(BX) = K_O\,(B)\,(X),$$

where K_O is the equilibrium constant of the O_2 reaction. Combining this with (IV) and (V) we find,

$$\frac{y}{1-y} = \frac{K_3}{K_1}(K_O)^n(X)^n = K(X)^n \text{ say}$$

which is the ordinary equation to the O_2-dissociation curve. Similarly, if the gas X be CO only, we obtain identically the same equation with another constant K. Furthermore, CO_2 may be assumed (see below) to act upon the combination by capturing some of the Na satisfying the spare linkage on the protein molecule, and so reducing the value of K_3. Its effect therefore would be identical in the two cases of O_2 and CO, as observed.

Finally, if the gas X be a mixture of O_2 and CO, the equation (II) becomes,

$$B + O_2 \rightleftarrows BO_2$$

$$B + CO \rightleftarrows BCO$$

with the result,

$$(BO_2) = K_O\,(B)\,(O_2)$$

$$(BCO) = K_{CO}\,(B)\,(CO)$$

from which,

$$(BX) = (BO_2) + (BCO) = (B)\,[K_O(O_2) + K_{CO}\,(CO)].$$

Combining this with (IV) and (V) we find,

$$\frac{y}{1-y} = \frac{K_3}{K_1}[K_O\,(O_2) + K_{CO}\,(CO)]^n.$$

Thus the total amount of haemoglobin saturated with *gas* is

$$y = \frac{K_3/K_1\,[K_O(O_2) + K_{CO}\,(CO)]^n}{1 + K_3/K_1\,[K_O(O_2) + K_{CO}(CO)]^n}$$

of which a fraction,

$$K_O(O_2)/[K_O(O_2) + K_{CO}\,(CO)]$$

will be saturated with O_2, and a fraction,

$$K_{CO}\,(CO)/[K_O(O_2) + K_{CO}\,(CO)],$$

with CO. These relations are identical with those shown above (p. 578) to satisfy all the observed facts. Further, with regard to the action of CO_2, if the amount of O_2 and CO be sufficient to saturate the haemoglobin completely, it is clear that the rectangular hyperbola giving the distribution of the haemoglobin between CO and O_2 is affected only by the constants K_O and K_{CO}, and not at all by the constants K_3 and K_1. But the action of CO_2, on this theory, lies in its tendency to seize upon the Na satisfying the spare linkage of the gas-saturated haemoglobin complex, *i.e.* in its effect on the constant K_3: thus the presence of CO_2 should have no effect on the relative distribution of

saturated haemoglobin between CO and O_2, which depends only upon the constants K_O and K_{CO}. This again is in accordance with observation.

There are certain other facts which, although indirectly, provide independent confirmation of the theory. There is no doubt that the combination between the protein and the iron-containing part, is a very loose one, and it is known that chemical agents (*e.g.* potassium ferricyanide), or bacteria, or a high temperature, act much more readily on haemoglobin when uncombined with gas, than when combined, and much more readily on oxy- than on CO-haemoglobin [see *e.g.* Hartridge, 1912, 2]. One would expect the active agent to work more readily on dissociated molecules. But the presence of a large proportion of unsaturated Hb would lead (see equation (I)), by the laws of mass action, to a large proportion of free dissociated molecules. The presence of O_2 would eliminate these molecules by combination, as in equation (II). The presence of CO however, even at low pressures, would eliminate them much more effectively, since a small breakdown of the gas-combined compound would immediately and enormously raise the effective combining pressure of the CO, leading to an almost complete absence of the dissociated molecules of haemoglobin.

A further point of interest arises in connection with the effect of temperature. Hartridge [1912, 1] found a relatively small, but definite, effect of temperature upon the position of equilibrium of the reaction,

$$\text{Hb O}_2 + \text{CO} \rightleftarrows \text{Hb CO} + \text{O}_2.$$

Taking his figures, and employing the equation

$$K = K_0 e^{-q(T-T_0)/2TT_0}$$

to determine the heat of reaction, we find

$$q = 5240 \text{ calories per g. molecule.}$$

This is some 19 % of the value found by Barcroft and Hill [1910], by the same method, for the heat of reaction of one gram-molecule of oxygen with dialysed haemoglobin, and about 50 % of the value similarly found in hitherto unpublished experiments by Brown and Hill in the case of whole blood. Recent experiments however by Hartridge [1921] have shown that his earlier observations were in error, by not allowing for a natural shift of the α-band with temperature, and suggest that the effect of temperature on the position of equilibrium of O_2 and CO with Hb is really *nil*, or practically *nil*, a conclusion which confirms an earlier observation of Haldane and Lorrain Smith. In that case the heat of reaction of

$$\text{Hb O}_2 + \text{CO} \rightarrow \text{Hb CO} + \text{O}_2$$

is very small, or nothing. Thus, even though CO has hundreds of times the affinity for Hb that O_2 has, its heat of reaction is almost exactly the same: this makes all the more likely the hypothesis that in the recombination of the protein with the gas-saturated iron-containing molecule no effect is exerted by the actual chemical nature of the gas, but only by the fact that a

free affinity is satisfied; combination with either gas has reduced the total energy of the molecule by identically the same amount, and the protein is indifferent as to which gas has done it.

The effects of acids and CO_2 *on the dissociation curve.*

It has been known for some time, chiefly as the result of the work of Barcroft, that the addition of acid to blood has a large effect on the dissociation curve, and recently Adair and Henderson have shown empirically that $1/K$ is a linear function of the CO_2 pressure. It is possible to deduce these interesting and important facts from the theory as follows: we suppose that the haemoglobin in blood is $H.(Hb)_n$, a very weak acid, while the oxyhaemoglobin is $H.(HbO_2)_n$, a stronger acid [Christiansen, Douglas and Haldane, 1914]. The following equilibria then co-exist inside the corpuscle (small letters represent concentrations, and B represents alkali, Na or K, inside the corpuscle):

$$\underset{}{H(Hb)_n + B' + OH' \rightleftarrows B(Hb)_n + H_2O} \qquad \text{.....(A)}$$
$$\underset{(1-y-z-u)\quad (b)\quad (f)\qquad (y)}{}$$

$$\underset{}{H(Hb)_n + nO_2 \rightleftarrows H(HbO_2)_n} \qquad \text{.....(B)}$$
$$\underset{(1-y-z-u)\quad (x)\qquad (z)}{}$$

$$\underset{}{B(Hb)_n + nO_2 \rightleftarrows B(HbO_2)_n} \qquad \text{.....(C)}$$
$$\underset{(y)\quad (x)\qquad (u)}{}$$

$$\underset{}{H(HbO_2)_n + B' + OH' \rightleftarrows B(HbO_2)_n + H_2O} \qquad \text{.....(D)}$$
$$\underset{(z)\quad (b)\quad (f)\qquad (u)}{}$$

From these equations we have,

From (A), $$\frac{y}{1-y-z-u} = k_1 fb.$$

From (C), $$u/y = k_3 x^n.$$

From (D), $$u/z = k_4 fb,$$

where k_1, k_2, k_3, and k_4 are the equilibrium constants of the several reactions.

From these we can deduce that,

$$\frac{z+u}{1-(z+u)} = x^n k_3 \frac{(1+1/k_4 fb)}{(1+1/k_1 fb)}.$$

But $(z + u)$ is the O_2-saturated fraction of the haemoglobin, $[1 - (z + u)]$ the unsaturated fraction, so that this equation is the same as the ordinary "Hill's equation" of the dissociation curve, with a K equal to $k_3(1+1/k_4 fb)/(1+1/k_1 fb)$.

Hence, $$\frac{1}{K} = \frac{1}{k_3} \frac{(1+1/k_1 fb)}{(1+1/k_4 fb)}.$$

Now oxyhaemoglobin is a much stronger acid than reduced haemoglobin, or in other words, k_4 is much greater than k_1. But, if k_4 be large, $1/k_4 fb$ will be small, and we may neglect it in comparison with the other terms. Assuming that we may neglect it we obtain immediately,

$$\frac{1}{K} = \frac{1}{k_2}(1 + 1/k_1 fb).$$

Let k be the dissociation constant of water: then $f = (OH') = k/C_H$: hence

$$\frac{1}{K} = \frac{1}{k_3}(1 + C_H/kk_1b).$$

Thus $1/K$ depends upon k_3, k, k_1, which are chemical constants, independent of everything presumably except temperature, and upon C_H/b, the ratio of the hydrogen to the basic ion concentration. Thus $1/K$ will be increased, and the dissociation curve shifted to the right, by an increase in the hydrogen ion concentration of the contents of the red blood corpuscle. This agrees with the experimental facts. A study of the actual quantities involved shows that C_H/kk_1b is usually so large compared with 1 that the equation may be written, without serious error,

$$1/K = C_H/kk_1k_3b.$$

This also agrees with the facts. Barcroft and Peters [1914], and Donegan and Parsons [1919], have shown that the relation between $\log K$ and the P_H ($-\log C_H$) of the plasma is a straight line (the so-called "P–B line"). This means that $1/K$ is proportional to some power of C_H, and since this straight line is usually inclined at 45° to the axes this power is usually the first: thus $1/K$ is proportional to $(C_H)^m$ where m is generally unity. This agrees with the above deduction. If we suppose the haemoglobin molecule to be capable of reacting with more than one molecule of base, *e.g.* according to the scheme,

$$H_2(Hb)_n + 2B^{\cdot} + 2OH' \rightleftarrows B_2(Hb)_n + 2H_2O$$

then $1/K$ will be found to depend not upon the first but upon some other power of the hydrogen ion concentration: this may account for "P–B lines" inclined at an angle other than 45° to the axes. According to the equation $1/K$ varies inversely with b the concentration of basic ions. Thus it is not the hydrogen ion concentration alone which determines the dissociation curve: it is *the ratio, inside the corpuscle, of the hydrogen ion to the basic ion concentration. These two ions must be regarded as competing with one another for possession of the haemoglobin.* One individual may have a higher $Na^{\cdot}$ or $K^{\cdot}$ concentration inside his corpuscles than another, so that at a given C_H their dissociation curves will be different: this may explain certain well-known differences between individuals or species.

In the presence of CO_2 the C_H *outside* the corpuscle is given by the relation $C_H = \alpha(H_2CO_3)/(HCO_3')$, where (H_2CO_3) is the concentration of dissolved CO_2 in the plasma, (HCO_3') that of bicarbonate ions, and α is the dissociation constant of carbonic acid. If we assume that the C_H of the plasma is the same as, or is proportional to, the C_H of the corpuscular contents, we may substitute this value of C_H, or a number proportional to it, in the above equation, so obtaining,

$$\frac{1}{K} = \frac{1}{k_3}[1 + \alpha(H_2CO_3)/kk_1b(HCO_3')].$$

Thus, on the theory, $1/K$ is a linear function of the CO_2 pressure, in direct agreement with Adair's [1921] experimental results. Moreover, this form of

the equation gives some meaning to the constants. The constant $1/k_3$ (Adair's constant b) is the inverse of the equilibrium constant of the reaction

$$B(Hb)_n + nO_2 \rightleftarrows B(HbO_2)_n.$$

Being the constant of a definite chemical reaction we should expect to find it invariable except for a change of temperature. Adair finds it practically unaffected, in general, by dyspnoea. On the other hand the constant

$$a/kk_1b\,(HCO_3')$$

(Adair's a/b) is simply a certain constant divided by the product of the concentrations (i) of basic ions inside the corpuscle and (ii) of bicarbonate ions outside. In dyspnoeic cases Adair found a much higher value of his a, and about the same value of his b, *i.e.* a higher value of a/b. Thus, on the theory,

$$a/kk_1b\,(HCO_3')$$

is increased in dyspnoea. But a/kk_1 should be invariable (except for a change of temperature) being derived from the constants of definite chemical reactions. Hence any increase in Adair's a/b must be due to an increase in $1/b\,(HCO_3')$. But in dyspnoea we should expect HCO_3', the amount of free dissociated bicarbonate present, to be reduced, and $1/b\,(HCO_3')$ to be increased. This agrees therefore with Adair's experimental results. Thus the increase, in dyspnoea, of the ratio a/b of Adair's constants a and b may be credited simply to a reduction in the amount of bicarbonate in the plasma, or possibly to a reduction in the concentration of basic ions inside the corpuscle: while the linear relation between $1/K$ and the CO_2 pressure is due to the combinations of reduced- and oxy-haemoglobin with the base which is being competed for simultaneously by the CO_2. Thus the effects of CO_2 upon the dissociation curve of blood can be attributed simply to physico-chemical reactions between the haemoglobin regarded as an acid, the sodium bicarbonate, and the CO_2. It would be interesting to pursue the subject further by an experimental investigation of the effects of temperature, etc., upon the several reactions involved.

Summary.

A theory is suggested to explain:

(*a*) the S-shaped dissociation curve of oxy-haemoglobin in the presence of salts, and the similar curve of CO-haemoglobin;

(*b*) the identical effects of CO_2 upon these curves;

(*c*) the rectangular hyperbola relating CO-saturation to CO-pressure, or O_2-saturation to O_2-pressure, in blood or haemoglobin fully saturated with a mixture of O_2 and CO;

(*d*) the absence of any effect of CO_2 upon these curves;

(*e*) the paradoxical relationship between CO-saturation and O_2-pressure, or between O_2-saturation and CO-pressure, in blood or haemoglobin only partly saturated with a mixture of O_2 and CO.

It is shown also that the effects of acid, and of CO_2, upon the dissociation curve of blood, can be deduced from the hypothesis that the available alkali inside the corpuscle is competed for by the oxy-haemoglobin, the reduced haemoglobin, and the acid or CO_2, the first being a far stronger acid than the second. The so-called "P–B" line (the relation between log K and P_H), and the Adair-Henderson line (the relation between $1/K$ and CO_2 pressure) are thereby explained. The constant $1/K$ is shown to depend upon the ratio of the hydrogen to the basic ion concentration inside the corpuscle. This may explain the differences in the dissociation curves of different individuals.

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LXXII. THE BACTERICIDAL ACTION OF THE QUINONES AND ALLIED COMPOUNDS.

By GILBERT THOMAS MORGAN and EVELYN ASHLEY COOPER.

From the University of Birmingham.

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Wurster [1889] and Raciborski [1907] first showed that various quinones gave red colorations with proteins, proteoses, and amino-acids. Meunier and Seyewetz [1908] furthermore showed that hide could be tanned by quinol in the presence of oxygen and alkali or by benzoquinone itself, and suggested that the tanning process was due to the reaction between this quinone and the amino-groups of the tissue. Fischer and Schrader [1910] found that qiunones reacted with ethylglycine with the formation of quinol and a substance analogous to di-anilinoquinone.

One of us [Cooper, 1913] confirmed the observations of Wurster and Raciborski and investigated in more detail the relations of *p*-benzoquinone to proteins. It was found that not only were proteins coloured permanently red by treatment with solutions of this quinone, but other physical properties, *e.g.* solubility and precipitability, were also changed, and evidence was adduced that the quinone reacted as in the case of formaldehyde with the amino-groups of the proteins. Shortly afterwards Scharvin [1913] also observed that various proteins were dyed by benzoquinone and its homologues, and came to the same conclusion as the former workers in regard to the nature of the dyeing process. Anthraquinone and phenanthraquinone were without action.

Suida [1913] also found that wool was dyed by solutions of benzoquinone, toluquinone and 3:6-*o*-xyloquinone in acetic acid solution, but not by 2:5-*p*- and 2:5-*m*-xyloquinones, thymoquinone, anthraquinone or phenanthraquinone. He pointed out that the quinones active in dyeing all contained the —CO—CH=CH—CO— group and concluded that they reacted with the amino-constituents of the wool.

These facts themselves suggest that quinones would be powerful germicides and Thalhimer and Palmer [1911] found (apart from these considerations) that benzoquinone greatly exceeded phenol, cresol, quinol, and other substances in germicidal power, this being confirmed by Cooper [1912, 1].

A further study of the efficacy of the quinones and their derivatives as disinfectants has since been made with the object of discovering substances

suitable for use as germicides in the living organism, and the results so far obtained are set out in the present paper. First of all, however, reference should be made to certain observations of theoretical interest, bearing on the mechanism of the reaction which proceeds between quinones and proteins.

I. The Relations of Quinone to Proteins.

(*a*) *General Observations.*

As stated in a previous paper [Cooper, 1913] when gelatin was immersed in aqueous *p*-benzoquinone solutions, it became permanently red and insoluble in water. Egg-albumin suspensions also gave a red coloration, but the protein was not precipitated. Subsequently, however, it was found that if the albumin solution were mixed with strong solutions of this quinone (0·5 to 1 %) and the mixture left for several days, a red flocculent nitrogenous precipitate separated. This product was insoluble even in large volumes of water and alcohol, and was not decolorised by boiling in either medium. Unlike albumin coagulated by heat, or phenol, the precipitate was insoluble in hot anhydrous phenol or *m*-cresol and in acid, and was only slowly dispersed by alkali. The dispersion was destroyed by the addition of acid and by dilute ammonium sulphate, saturated solution of magnesium sulphate and sodium chloride, as is the case with albumin denaturated by heat or phenol.

The effect of some of the homologues of benzoquinone has also been studied. Toluquinone also gave intense red colorations with gelatin, egg-albumin, and caseinogen; xyloquinone and thymoquinone gave however no colorations.

Equal amounts of washed dry gelatin were immersed at room temperatures in equimolecular solutions of *p*-benzoquinone, toluquinone, xyloquinone, and thymoquinone (0·023, 0·037, 0·04, 0·05 % respectively). Immersion for less than 24 hours in the benzoquinone solution was sufficient to render the gelatin intensely red and insoluble in water. The gelatin suspended in the toluquinone solution for the same length of time was coloured less intensely and even after six weeks was not rendered insoluble. The gelatin immersed in the solutions of the higher homologues was unaffected. From these observations it seems that as the homologous series is ascended the reactivity of the quinones towards proteins diminishes; in the case of the phenols and alcohols, on the other hand, it was found [Cooper, 1913] that their protein-precipitating power increased as successive homologues were considered.

(*b*) *The removal of quinone from solution by proteins.*

The experiments described in this section were carried out with the object of investigating the influence of concentration and other factors upon the amount of quinone removed by proteins from aqueous solution.

Materials used. The proteins used were gelatin, egg-albumin and caseinogen.

Gelatin. The Swiss Gold Label brand was used. The gelatin was washed in running water for 24 hours, drained, air-dried and then cut into small slips and dried at 110°.

Egg-albumin. Crystalline egg-albumin prepared by the method of Hopkins and Pinkus [1898] was used. The crystalline protein was dialysed in running water and employed as a colloidal suspension.

Caseinogen, prepared according to Hammarsten, was employed. It was dried at 110° before use.

Estimation of quinones. The method used, which was that devised by Valeur for *p*-benzoquinone, and its homologues and derivatives, depends on the fact that a quinone sets free iodine from a mixture of hydrochloric acid and potassium iodide.

Experimental Methods.

Definite amounts of a protein were immersed in equal volumes of quinone solutions of known strength. At stated times the amounts of quinone left in the solutions were again estimated.

(*a*) *Experiments with precipitated caseinogen.*

The dialysing method of Moore and Bigland [1910] was employed, the protein being restricted to one region of the water-phase by means of parchment paper, so that the content of quinone in the aqueous solution could be accurately determined.

It was found that the quinone dialysed rapidly and was not taken up by the parchment. With phenol under these circumstances equilibrium was attained at 20° within 40 hours [Cooper, 1913], but with quinone, the concentration in the water-phase continued to fall after a much longer period and equilibrium was not reached in 10 days. For this reason *dispersed* proteins were subsequently used.

(*b*) *Egg-albumin* (*colloidal solution*).

Ten cc. of a dialysed suspension (18 %) were placed within the dialysing bag at 20° and 25 cc. of benzoquinone solution outside.

Table I.

Initial strengths of quinone solution			1. 0·284 %
,,	,,	,,	2 0·284
,,	,,	,,	3. 0·192
,,	,,	,,	4. 0·192

The concentration of quinone continued to fall during four weeks, until by the 30th day no quinone could be detected. In the case of phenol, however, the maximum amount was absorbed by the protein within 24 hours [Cooper, 1912, 1] in accordance with the partition-law. From the above results it is found that 1·8 g. of albumin removed 0·099 and 0·050 g. of quinone respectively from the solutions of 0·284 and 0·192 %. The amounts of phenol absorbed by

the same weight of protein from the same initial concentrations were 0·012 and 0·006 g. [Cooper, 1913], (calculated from partition-coefficients). Quinone was thus removed by egg-albumin in much greater amount than phenol from the same concentration.

(*c*) *Gelatin.*

As at 20° gelatin is not appreciably soluble in water, in the following experiments the dialyser was dispensed with.

Table II.

Weight of protein 2 g. Volume of quinone solution 100 cc. 20°.

Initial concentrations	1.	1·04 %
,, ,,	2.	0·52
,, ,,	3.	0·26

A fall in quinone concentration was again observed to continue for several weeks, and at the end of 50 days no quinone was left in solution. The uptake of phenol by gelatin however was complete within two minutes [Cooper, 1913].

The results show that 2 g. of gelatin removed 1·04, 0·52, 0·26 g. of quinone respectively from the three solutions. The amounts of phenol removed by the same amount of protein from the same concentrations of phenol, on the other hand, were 0·06, 0·03, 0·015 g. respectively [Cooper, 1913], (calculated from partition-coefficients). As in the case of albumin, more quinone than phenol was thus removed by gelatin from the same concentration of these substances. Furthermore, while the absorption of phenol by gelatin and albumin was reversible, it was not possible to recover any quinone from the proteins even after boiling in water.

Experiments were next carried out with smaller amounts of gelatin with the object of determining an equilibrium-point.

Table III.

(*a*) Weight of gelatin 0·5 g. Vol. of quinone solution 150 cc. Temp. 20°:

Initial quinone concentration	Quinone concentration in water-phase after 8 weeks	Total amount of quinone removed by the protein after 8 weeks
1. (*a*) 0·620 %	0·198 %	0·633 g.
(*b*) 0·620	0·198	0·633
2. (*a*) 0·310	0·106	0·306
(*b*) 0·310	0·106	0·306
3. (*a*) 0·124	0·033	0·136
(*b*) 0·124	0·033	0·136
4. 0·062	0·009	0·080
(*b*) Weight of gelatin 0·25 g.		
1. 0·620 %	0·320 %	0·450 g.
2. (*a*) 0·310	0·176	0·201
(*b*) 0·310	0·176	0·201
3. 0·124	0·062	0·093
4. 0·062	0·029	0·050

From all the foregoing concentrations the gelatin continued to remove quinone for several weeks, and equilibrium was not even attained after

eight weeks. It is seen from the table that gelatin removed or reacted with very large amounts of quinone, 0·25 g. of the protein for example removing from an initial concentration of 0·62 % as much as 0·45 g. of quinone. The results in eight weeks also show that the amount of quinone disappearing depends upon the initial concentration, being in fact approximately proportional to the concentration.

The observations so far strongly suggest that a slow chemical reaction proceeds between quinone and proteins, especially as Benedicenti [1897] found that formaldehyde, which reacts chemically with proteins, is gradually removed by the proteins from aqueous solutions for several weeks, until finally a maximum uptake is indicated.

Some washed gelatin and dialysed egg-albumin were next added to aqueous solutions of quinone and when all the quinone had disappeared, the solutions were extracted with ether. It was possible in each case to detect quinol in the ethereal extract. This suggested that quinone was behaving as a peroxide and that substances were formed in the interaction of proteins and the quinone analogous in constitution to the anilino-derivatives in the case of ethylglycine. It does not exclude, however, the possibility of the alternative reaction in which quinone behaves as a diketone as formerly suggested [Cooper, 1913]. The results show conclusively that quinone reacts with proteins, and that the interaction is of some complexity.

II. The Bactericidal Action of the Quinones.

The remarkably high bactericidal power of quinone has already been referred to in previous communications [Cooper, 1912, 2, and 1913] and the evidence therein presented suggested that this reactivity was associated with a chemical action on certain constituents of the bacterial protoplasm. As benzoquinone exhibits dynamic isomerism and is capable of reacting as either a diketone or a peroxide, it was felt that a further study of this very reactive substance and its homologues from the point of view of their bactericidal power and relation with proteins would be of great interest.

Table IV. *Carbolic acid coefficients.* Temp. 20°.

	B. typhosus		*Staphylococcus py. aur.*	
Disinfectant	15 mins.	30 mins.	15 mins.	30 mins.
p-Benzoquinone	190·0	—	12·6	21·0
„	80·0	—	10·0	—
p-Toluquinone	20·0	70·0	<4·0	3·25
Xyloquinone	<19·0	<19·0	—	—
Thymoquinone	<19·0	<19·0	—	—
Phenol	1·0	1·1	1·0	1·0
o-Cresol	2·6	—	2·1	—
m-Cresol	2·6	—	2·0	—
p-Cresol	2·6	—	2·4	—
Thymol	25·0	—	—	—
Quinol	1·0	—	—	—
Formaldehyde	0·5	—	0·3	—
Benzaldehyde	10·0	—	10·0	—

For this purpose bactericidal power has been measured in terms of carbolic-coefficients, determined by the method of Chick and Martin [1908] in the absence of organic matter.

It was thought possible that the high germicidal power of the quinones was due to the inhibitory action of traces carried over to the broth-tubes during the process of sub-culturing. To ascertain if this were the case, the organisms were grown in broth at 37° in the presence of definite concentrations of the quinones. The following results were obtained:

Table V.

Benzoquinone concentration	Growth and no growth	Toluquinone concentration	Growth and no growth
1 in 10,000	–	1 in 10,000	–
1 in 100,000	+	1 in 25,000	+
1 in 1,000,000	+	1 in 100,000	+
1 in 10,000,000	+	1 in 1,000,000	+

The results show that no marked inhibitory action took place, and it may be concluded that this factor did not interfere in the carbolic coefficient determinations.

Several matters of interest arise from the results, set out in Table IV. In the first place, it is seen how extraordinarily selective benzoquinone was found to be in its bactericidal action. Not only were there wide differences in the carbolic coefficients determined with distinct organisms, but a considerable divergence also existed when different cultures of the *same* organism were employed. Although selective action is well known in disinfection, the differences observed are not usually so marked as found in the case of benzoquinone. Whether these remarkable differences are due to specific action upon the bacterial protoplasm, or to selective permeability are matters for further investigation. The results also show that the bactericidal power of benzoquinone was enormously greater than that of phenol, and quinol. The bactericidal power of the quinones however diminished as the homologous series was ascended, while in the case of the phenols there was a marked increase. It is thus seen that while toluquinone was still superior to *o*-cresol, the difference was not so great as in the case of the first members of the series. Furthermore, thymoquinone was actually inferior to thymol in germicidal efficiency. Morgan and Cooper [1912] previously found that there was a rise in bactericidal power in ascending an homologous series not only in the case of phenols, but also in the case of the monohydric alcohols and the aliphatic and aromatic amines; heptylamine, for example, possessing the very high carbolic coefficient of 27.

The behaviour of the quinones is thus exceptional and calls for some special explanation.

It is well known that benzoquinone exhibits dynamic isomerism, while the homologous quinones exist mainly in one (peroxide) form. The superiority of benzoquinone in bactericidal efficacy may therefore be due to the continual liberation of its molecules in the nascent reactive condition.

Determinations of the bacterial power of certain aliphatic ketones were next carried out, and the results are tabulated below.

Table VI. *Carbolic acid coefficients.*

Substance	Organism *B. typhosus*	Substance	Organism *Staphylococcus py. aur.*
Benzoquinone	190·0	Quinone	12·60
Acetylacetone	0·20	Acetone	0·07
Formaldehyde	0·50	Diacetyl	0·30
		Formaldehyde	0·30
		iso-Propyl alcohol	0·04

It is seen that the three ketones: acetone, diacetyl, and acetylacetone, are remarkably feeble germicides, and are not only less efficacious than quinone, but even weaker than phenol and formaldehyde.

Acetone, also, is not appreciably stronger than the secondary alcohol, *iso*-propyl alcohol, from which it is derived.

It is also seen that the aliphatic β-diketone acetylacetone which yields a tautomeric "*enolic*" form is much feebler in bactericidal power than benzoquinone which forms a *peroxide* isomeride.

Summary.

1. When proteins are added to solutions of *p*-benzoquinone, the latter slowly disappears, and equilibrium is not attained even after several weeks. Quinol can be detected in the course of the reaction.

2. *p*-Benzoquinone thus appears to react chemically with proteins as a peroxide, and differs fundamentally from the phenols, which are distributed between water and proteins according to the partition-law and behave merely as protein precipitants.

As previously pointed out, *p*-benzoquinone may possibly also react as a ketone with proteins, a simple condensation taking place as in the case of formaldehyde.

3. Benzoquinone possesses a remarkably high bactericidal power, being from 80–190 times as efficacious in destroying *B. typhosus* as quinol and phenol.

4. The bactericidal power of the quinones diminishes as the homologous series is ascended, while with the phenols, alcohols, and amines the opposite is the case. Consequently thymoquinone is less efficacious as a germicide than the corresponding phenol, thymol.

5. The aliphatic ketones, acetone, diacetyl, and acetylacetone are much less efficient as germicides than benzoquinone, or even phenol and formaldehyde.

6. Acetylacetone exists in two tautomeric forms, ketonic and enolic, and it is thus seen that a diketone yielding an enolic form is much feebler in bactericidal efficacy than one, such as benzoquinone, giving a peroxide form.

Conclusion.

Further investigations must be carried out before the mechanism of the bactericidal action of benzoquinone is fully understood, but so far the results show that the extraordinarily high bactericidal power of this substance is associated with its property of exhibiting dynamic isomerism and is probably due to the chemical interaction of the *nascent* peroxide molecules with certain protein-constituents of the bacterial protoplasm.

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LXXIII. EXPERIMENTAL STUDIES ON CELLULAR MULTIPLICATION.

I. THE MULTIPLICATION OF ISOLATED INFUSORIA.

By THORBURN BRAILSFORD ROBERTSON.

From the Department of Physiology and Biochemistry of the University of Adelaide, South Australia.

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Statement of the Problem.

Those unicellular organisms which multiply by fission are, as Wassermann and others have pointed out, potentially immortal, since the protoplasm of the parent is contained in and shared between the daughter-cells. Furthermore, in the presence of unlimited food and environmental conditions which are otherwise suitable they are possessed of conceivably unlimited capacity for reproduction.

The actual conditions which attend the life of these organisms, however, never permit the full realisation of their capacity for multiplication, for even in the absence of any other check to unlimited reproduction, the ratio of population to food supply must sooner or later attain a point at which the further synthesis of protoplasm is an impossibility, and all obtainable food materials are requisite, or perhaps insufficient, to maintain the existing population.

Notwithstanding the imaginable potentialities of a Protozoon, therefore, the actual phenomena which are displayed by a culture in a limited amount of culture medium are not unlike those which characterise the life-cycle of the interdependent cell-masses which compose the Metazoa. In the one case, as in the other, the synthesis of protoplasm, starting from a single cell, becomes more and more rapid as the number of cells increases, until a maximal reproductive rate, or rate of growth, is attained. From this point onwards, for reasons which are not as yet perfectly understood, the cellular reproductive capacity progressively wanes until the population has attained a maximum.

It will subsequently be shown that a Protozoon culture exhibits youth, age, and the phenomenon of senescence, that is, not merely the slowing down of multiplication which is age, but that diminution of capacity to multiply, even under suitable conditions and in the presence of abundance of foodstuffs, which is senescence. The salient characteristics of the life-cycle of the higher animals are therefore also displayed by a culture of infusoria, and although the individual Protozoon is potentially immortal, the society comprised in a

given culture is subject to the law of mortality which sets a term to the existence of more complex cell societies.

Those factors which are fundamental to and reside in the nature of protoplasm itself must operate alike in a Protozoon culture and in the specialised cell-aggregates which form the Metazoa. A study has therefore been undertaken of the various factors which determine reproductive rate in cultures of infusoria, with a view to the application of information so obtained to the interpretation of the phenomena of growth in higher animals.

There are especial advantages in approaching the problems of growth from this angle, because the units which comprise a culture of infusoria are separable from one another without the slightest disturbance of the normality of their environment. The several units are alike, and their reproductive capacities may be directly ascertained by counting their descendants in a given period of time. Finally the entire cycle of development, from the single cell to the multitude of its descendants which are no longer able to increase, occupies a period of but a few days.

Methods of Investigation.

The species of infusorian employed in these experiments was *Enchelys farcimen* Ehr. It is an ovate or pyriform organism, finely ciliated throughout, the cilia being of uniform length over the greater part of the body, but somewhat longer in the oral region. The mouth is at the narrowed anterior extremity, directed somewhat to one side. In old and "starved" cultures the organism is usually about 50 μ in length and one-third as broad, but in well-fed cultures which are only 24 hours old, or even 48 hours old when for any reason development has been delayed, the organisms are much broader and of a pear-shaped or even cubical outline. The pear-shaped organisms measure from 50 μ to 70 μ in length and from 35 μ to 50 μ in breadth. Recently divided organisms are somewhat more than half the length of the fully grown individuals and globular or pear-shaped. Exactly similar changes of outline with abundance or scarcity of food have been noted by Jennings in cultures of *Paramoecium* [Jennings, 1910].

All of the organisms employed in these experiments arose, by repeated propagation of single individuals into fresh culture media, from two "wild" individuals originally isolated from a culture of chrysanthemums. The descendants from these two individuals have been separately propagated for over 200 generations, forming an "A" and a "B" series of cultures. Throughout this period no uniform difference whatever has been observed in the rates of multiplication of the two strains, the few instances in which individuals simultaneously isolated from "A" and "B" cultures yielded decidedly differing numbers of descendants in 24 hours being in every case traceable to differences in the histories of the parent cultures or in the treatment of the subcultures.

The culture medium employed was a hay infusion formed by heating five grams of chaffed hay in 100 cc. of tap water to boiling and then continuing

the heating on a boiling water-bath for 30 minutes. The infusion was filtered, 0·4 cc. of $N/10$ Na_2CO_3 was added to every 10 cc. of the filtrate, and the mixture usually employed as soon as it was cool, being made up afresh for each day's subcultures. This is subsequently referred to as "Normal Hay Infusion." In some experiments this fluid was allowed to stand at room temperature in a shallow covered layer, for from 24 to 72 hours, at the end of which period it is thickly populated by bacteria which have arisen from the spores which are not destroyed by boiling. This fluid was filtered through paper to free it from the zoogloeal masses floating upon its surface, the filtrate constituting what is subsequently referred to as "Bacterised Hay Infusion."

Subcultures were prepared by removing from the parent culture a minute drop, averaging about 0·002 cc. by means of a pipette drawn out to a fairly wide capillary bore. This drop, usually containing from ten to thirty individuals, was placed in the centre of a depression in a microscope slide and diluted by the addition of a larger drop, averaging about 0·04 cc. of the fresh culture medium. Another minute drop was abstracted from this mixture and the process repeated until a single individual was obtained. Since it is possible to overlook an individual swimming close to the curved edge of a drop, and abnormal results in some of the earlier experiments were traced to this origin, the practice was adopted of invariably *re-isolating* the individual by diluting the drop in the manner indicated and abstracting small samples until one was found again to contain the single individual. This drop, measuring about 0·002 cc. was then diluted by the addition of two large drops or 0·08 cc. of culture fluid, forming the final subculture. It will be observed that even if the first small drop isolated from the parent culture chanced to contain but a single individual, the final dilution of the parent culture fluid in the new subculture could not be less than 1 in 800 and, of course, it was usually much greater.

The slide containing the culture was placed upon a pad of filter paper, soaked in water, in a petri dish, the edges of the dish being vaselined to prevent evaporation.

The food of infusoria consists, not of the soluble substances which are present in the infusion, but of the bacteria which subsequently develop therein. Hence the factor which determines the food supply is the degree of bacterial infection in the culture fluid. For reasons which will be evident in the sequel, a culture heavily infected with bacteria may cause very rapid multiplication of infusoria. Herein lies one of the main difficulties in standardising the culture medium employed. For if the culture be too nearly sterile infusorial development is very slow, and if it be allowed to accumulate a large bacterial population before being employed the relative abundance of bacteria in different infusions may conceivably vary very greatly from time to time. It was felt that the nearest practicable approach to standardised conditions was afforded by employing for each day's subcultures freshly-prepared "Normal Hay Infusion," containing, presumably, comparable numbers of bacterial

spores. In the course of the experiments it was found necessary, however, to sterilise the glassware employed to contain the infusion, the pipettes employed for isolation and dilution and the slides upon which the cultures were grown. Otherwise a film of bacteria on the surface of the glass may occasionally lead to irregular results. The technique employed was essentially similar to that described by Jennings [1913, 2]. Containers, measuring cylinders, etc., were rinsed with ether which was subsequently washed out with repeated jets of distilled water.

No attempt was made, in the majority of the experiments, to maintain a constant temperature, because the thermal death-point for these infusoria is so low that for the greater part of the year in South Australia room temperatures represent approximately optimal conditions.

The organisms were isolated and counted under a magnification of 64 diameters, employing a moderately bright, artificially illuminated field, against which they appeared relatively opaque.

The Influence of the Age of the Parent Culture upon the Multiplication-rate in Subcultures.

The rate of multiplication of individuals isolated from cultures over 48 hours old is very much slower than the rate of multiplication of individuals which are isolated from younger cultures. This fact is displayed in Table I and in the curve (Fig. 1) which summarise the various results obtained during the period of eight months throughout which these cultures have been the subject of experimentation. The temperatures were not uniform and for this reason there appears little utility in calculating the probable errors of the averages obtained, since the observed variation of the results depended, not merely upon the inherent variability of the reproductive power of the cells themselves, but also, in part, upon varying extraneous factors. Nevertheless the results are sufficiently uniform to display the essential phenomena, and the conditions of propagation were alike for each age of parent culture, so that the averages obtained in each class are comparable with one another.

The initial rapidity with which the reproductive capacity of isolated individuals falls off with the age of the parent culture is very remarkable. This rapid loss of reproductive power during the first 48 hours is succeeded by a slow but definite further loss during the following 48 hours. The slight recovery indicated in the last column of Table I (parent cultures 5 to 9 days old) may be merely apparent and due to the variability of the individual results and an insufficient number of observations to establish a true average. I am inclined to believe, however, that this increase is not apparent but real, and that the starvation to which the organisms in these very old cultures have been subjected renders them exceptionally sensitive to certain chemical conditions which, as we shall see, favour rapid multiplication. Experimental data bearing upon this point are not as yet sufficiently numerous, however, to enable me to affirm this view with certainty.

Table I. *Cultures in Normal Hay Infusion.*

Number of individuals produced in 24 hours from a single individual isolated from parent cultures of the following ages:

1 day old		2 days old		3 days old		4 days old	5 to 9 days old
16	14	4	8	8	2	2	2
16	11	1	12	7	2	2	2
33	71	4	8	2	2	2	4
32	46	28	8	2	8	2	2
64	31	10	24	1	4	2	2
93	18	8	2	1	2	3	1
105	14	9	4	1	1	1	4
96	14	8	3	1	7	1	2
40	18	2	2	4	2	4	1
52	21	2	8	4	2	4	2
30	56	2	4	4	2	2	2
24	24	3	4	2	2	2	4
56	16	2	4	2	2	1	2
26		8	1	2	4	2	1
		6	1	1	4	1	8
		4	3	1	4	1	6
		4	1	1	2		
		4		1	3		
				1	1		
				1			
Averages:	38·4		5·9		2·6	2·0	2·8

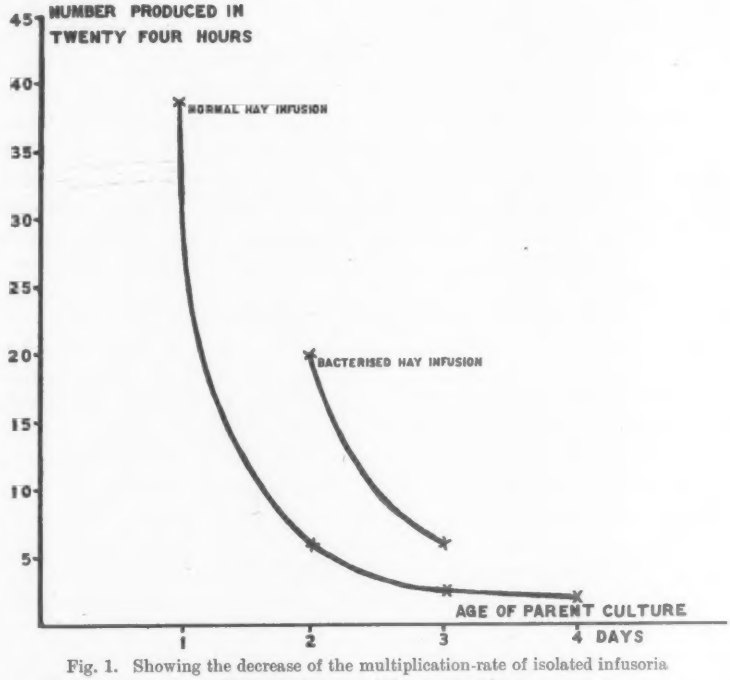

Fig. 1. Showing the decrease of the multiplication-rate of isolated infusoria with increasing age of the parent culture.

The loss of reproductive capacity in individuals isolated from old parent cultures is due to changes which have occurred in the organisms themselves, and not to any retardative substances associated with the organisms in the parent culture and introduced together with the isolated individuals into the subculture. This is shown by the fact that repeated washing of the isolated individual in freshly-prepared hay infusion or in tap water or in faintly alkaline distilled water (2 cc. of $N/10$ Na_2CO_3 to every 100 cc. of water) does not restore its reproductive capacity. Moreover the parent culture fluid is abundantly capable of supporting and permitting reproduction of infusoria if those already inhabiting it are removed.

It is not easy to remove these infusoria from a culture by mere filtration through paper, because *Enchelys* is able to pass through the pores of even hardened filter paper. It was not desired to remove them by filtration through a Berkefeld filter, because this procedure would also remove the bacteria which form the food of the infusoria. But by heating to 50° C. for a few moments the infusoria are all killed and, having been immobilised, they are readily removed from the fluid by filtration through paper. The bacteria, at least the majority of them, are not killed by exposure to this temperature, and they pass freely through rapid-filtering paper. Now on subculturing single individuals into this fluid normal reproductive rates are observed, comparable to those observed in simultaneously prepared subcultures into bacterised hay infusion which has never been tenanted by infusoria. The following experiment illustrates this fact:

Bacterised hay infusion which had stood for two days at room temperatures was heated to 50° C. and then immediately cooled and filtered. Infusorial "mass cultures," two and five days old respectively, and densely inhabited by infusoria, were similarly heated to 50° C. and then filtered. Single individuals from the same parent cultures (A and B series), 48 hours old, were isolated into each of these culture media:

Culture number	Culture medium	Number of individuals after 24 hours
197 A	Bacterised hay infusion	8
197 B	" "	16
200 A	Filtrate from 2-day infusorial culture	8
200 B	" " "	11
201 A	Filtrate from 5-day infusorial culture	8

The origin of the loss of reproductive capacity in old cultures is therefore to be sought in the density of the population, and not in any failure of the culture fluid to supply necessary nutrients or in any accumulation of products adverse to the multiplication of infusoria. The most probable explanation of this fact would appear to be that the nutritive requirements for the maintenance of the numerous infusorial population in an old culture have overtaken the rate of the reproduction of bacteria, so that the bacterial population is consumed just as rapidly as it is produced and the infusoria are thereby maintained at a nutritive level but little above the level of starvation. When the infusoria are removed the bacteria are permitted an opportunity to multiply before the new infusorial population attains a sufficient density to check them. We may

infer, therefore, that a period of existence at a low nutritive level is the factor which is actually responsible for the loss of the reproductive capacity of infusoria in old cultures.

This interpretation of the phenomenon is supported by the direct observation that the bacterial population of old infusorial cultures is very small and speedily increases after removal of the infusoria, and it is furthermore supported by the frequently-repeated observation that it is the density of the infusorial population rather than the age of the culture in days which determines the reproductive capacity of its members. If for any reason such as lowered temperature, or a low reproductive capacity of the parent culture, development of infusoria in a subculture is retarded, then at two days the individuals isolated from it may display the reproductive capacity normal to individuals isolated from a 24-hour culture. In this way, subculturing alternately from old and young cultures, a rhythm of reproductive energy is readily induced and subcultures alternately exhibit populations of two and of from 16 to 32 individuals respectively in 24 hours after isolation. It is mainly to this fact that the wide variations of reproductive capacity are due which are displayed by individuals isolated from different parent cultures of equal age (cf. Table I).

The Influence of Preliminary Bacterisation of the Culture Medium upon the Multiplication-rate of isolated Individuals.

Bacterisation of the hay infusions by preliminary standing at room temperatures for from 24 to 72 hours remarkably stimulates the reproductive capacity of the isolated organisms, and it delays, but does not completely prevent, the loss of reproductive capacity which occurs with age of the parent culture (Table II and Fig. 1). Even in the presence of superabundance of food, therefore, the individuals isolated from old parent cultures suffer a definite loss of reproductive capacity which appears to be analogous to the senescence

Table II. *Cultures in Bacterised Hay Infusion.*

Number of individuals produced in 24 hours from a single individual isolated from parent cultures of the following ages:

2 days old		3 days old
34	16	8
29	8	8
28	31	1
34	60	2
35	8	3
17	8	2
26	7	8
17	4	3
8	11	16
11	16	9
8		
Averages: 19·9		6·0

which overtakes the tissue-cells of the higher Metazoa with advancing age. Here again, however, very old (*i.e.* starved) parent cultures may show a surprising recovery of reproductive capacity in bacterised infusions. Thus in one experiment individuals from cultures of the "A" and "B" strains which were no less than 8 days old had produced 33 and 31 individuals respectively 24 hours after isolation into a bacterised infusion.

It will be seen, on comparing the results enumerated in Tables I and II, that the reproductive capacities of individuals isolated into bacterised cultures are even more variable than the reproductive capacities of individuals isolated into normal hay infusion, initially sterile except for bacterial spores and the small contamination introduced together with the isolated infusorian. The same fact has previously been noticed by Jennings and it appears probable that the extent to which previous bacterisation of the infusion favours the multiplication of infusoria depends upon the ratio of bacterial to infusorial population and upon the period and temperature of the previous incubation. Experiments designed to ascertain the precise method of preliminary bacterisation which produces maximal acceleration of multiplication are at present in progress.

Heating the bacterised infusion to boiling-point, or even for 90 minutes on a boiling water-bath just before its employment, does not diminish the multiplication-rate of individuals isolated into it. The following is illustrative of many experiments:

Culture numbers 150 *and* 151. The parent culture was two days old. The hay infusion had stood for two days at room temperature:

Culture number	Number in subculture after 24 hours		Culture fluid
	A	B	
150	35	17	Bacterised hay infusion
151	26	17	Same, boiled before using

Evidently, therefore, living bacteria are not essential for the occurrence of the accelerated multiplication. Doubtless killed bacteria are not less available than living ones as sources of food materials, but although the final number of infusoria produced in a given culture is sooner or later determined by the number of bacteria, dead or living, which are present, yet the initial accelerative action of bacterised culture media remains very pronounced even after passage through a Berkefeld filter, which deprives it of bacterial content. This is illustrated by the following, typical of a number of experiments:

Cultures 234, 236 *and* 237. The parent culture was two days old. Cultures 234 (A and B) were isolated into normal hay infusion, 236 (A and B) into hay infusion which had been bacterised for two days and then filtered through a Berkefeld filter, 237 (A and B) into the same bacterised infusion, without filtration. The cultures were examined at frequent intervals and at the end of six hours division had occurred in each of the cultures 236 (A and B) while there was as yet no sign of division in the cultures 234 (A and B) or 237 (A and B). In 22 hours the numbers in cultures 234 and 236 were equal (4 in each) while the cultures 237 (A and B) were increasing more rapidly (8 and 5 respectively). In 28 hours the individuals in the unbacterised infusion numbered 8 and 14, in the filtered bacterised infusion 4 and 4, and in the unfiltered infusion 20

and 16. The acceleration due to bacterisation was not, in this instance, very pronounced, but it occurred first of all in the bacteria-free medium and only ceased to manifest itself when actual deprivation of nutriment compulsorily brought multiplication to a standstill. At 22 hours, when the numbers in the various cultures were approximately equal, the organisms inhabiting the filtered fluid were of the thin, actively-swimming, "starved" type, while those inhabiting cultures 234 and 237 were of the "fat" type and very sluggish in movement.

The interest of the above experiment lies especially in the observation of the moment at which the first division occurred, and the fact that it occurred earliest in the filtered bacterised medium. The following illustrates more strikingly the accelerative effect of soluble bacterial products, without confusion of the comparison by the secondary effects of unequal food supply:

Cultures 259 *and* 263. The parent culture was three days old. The hay infusion was bacterised by standing for 48 hours at 23° C. and then filtered through a Berkefeld filter. The filtrate was mixed with an equal volume of normal hay infusion:

Culture number	Number of individuals in subcultures in 24 hours		Culture fluid
	A	B	
259	1	1	Normal hay infusion
263	11	16	Same plus an equal volume of bacterised and filtered infusion

The accelerative effect of the bacterised infusion is not therefore dependent upon the presence of bacteria, whether living or dead, but upon the presence of soluble products arising from the inhabitation of the medium by bacteria.

It might be imagined that the change in the medium induced by an abundant population of bacteria consists in nothing more than an adjustment of the reaction to a more favourable hydrogen ion concentration. The quantity of Na_2CO_3 added to the normal hay infusion was, however, that which was found by repeated trials to yield the optimal reaction for multiplication. Saturation of the normal hay infusion with carbon dioxide increases the reproductive rate a little, but not to any extent comparable with the acceleration which may be produced by bacterisation. Moreover the accelerative agent is not volatile; it is not discharged from the bacterised fluid by boiling, nor can it be conducted by distillation into normal hay infusion.

Since the accelerative effect is independent of the supply of foodstuffs and may actually be evidenced by relatively starving organisms, the action of this substance presents analogies to the accessory rather than to the primary or calorific foodstuffs. The recent investigations of Williams [1919], Bachmann [1919], and of Funk and Dubin [1920] have shown that aqueous or acid alcoholic extracts of a variety of vegetable tissues, such as autolysed yeast or lucerne, contain a substance which has a marked accelerative effect upon the multiplication of yeast cells, and because of a rough parallelism which exists between the content of water-soluble accessory in such extracts and their stimulating effect upon the growth of yeast, it has been proposed to employ the increase in the rate of multiplication of yeast as a measure of the vitamin content of extracts. More extensive comparisons by Eddy, Heft, Stevenson and Johnson [1921], have shown, however, that the yeast tests

merely approximate the results of feeding tests on rats, the agreement being exact only within the range of dilutions between which the accelerative effect increases rapidly with concentration. Above this range the higher concentrations of the extract do not produce a corresponding increase in the reproductive rate of yeast, and an optimum concentration exists beyond which the curve of stimulation falls. No such phenomena are exhibited in feeding experiments with rats, so that the substance which affects the reproductive rate of yeast would not appear to be identical with the water-soluble vitamin. Furthermore Emmett and Stockholm [1920] have recently shown that the yeast-stimulating substance is thermostable, resisting boiling temperature for 90 minutes, while the water-soluble vitamin is destroyed under like conditions.

The substance in bacterised infusions which accelerates the reproductive rate of infusoria is similarly thermostable and may possibly be identical with the substance in autolysed yeast extracts, etc., which accelerates the reproductive rate of yeast. In its thermostability this substance (if, indeed, it is identical with any of the hitherto identified classes of food accessories), resembles the fat-soluble accessory rather than the water-soluble accessory, but until we possess a more exact knowledge of the relationship of the stimulative substance in bacterised hay infusions to the known food accessories on the one hand, and the yeast-stimulating substance on the other, I prefer for brevity of reference to employ the designation "X-substance."

Incidentally these results throw light upon the origin of an old misunderstanding. The original experiments of Engelmann [1876] and Maupas [1888, 1889] were held to have proved that asexual reproduction in infusoria could not continue indefinitely, because slide-cultures propagated repeatedly without conjugation died out, owing to total loss of reproductive capacity, in from 200 to 300 generations. On the other hand it was in some instances found that conjugation with a "wild" individual caused a remarkable access of vigour and reproductive capacity. Hence it was inferred that conjugation exerted a rejuvenating and invigorating effect upon the stock. Jennings [1913, 1], however, has demonstrated that if otherwise similar cultures be compared, those in which conjugation has occurred have actually an average reproductive capacity far *lower* than that of individuals which have never undergone conjugation. The offspring arising in conjugating cultures are, however, much more variable than those which arise by asexual reproduction, so that an occasional individual may chance to display a high reproductive rate. So far from exerting a rejuvenating effect upon the stock, conjugation brings about many abnormalities and greatly increases the death-rate among isolated individuals.

The loss of vigour in asexually propagated slide-cultures does not arise, therefore, from the absence of conjugation. According to Woodruff [1911] it is due to monotony of the environment, and he has succeeded, owing, he believes, to the fact that he repeatedly varied the culture medium, in propagating a culture of *Paramoecium* asexually, without any loss of reproductive

capacity, for over two thousand generations. In reading his description of the culture media employed, it appears very possible that the variation in the medium which, from time to time, resulted in re-invigoration of his cultures, may have been a favourable degree of preliminary bacterisation. He describes his culture media in the following words: "From May 1907, through January 1908 the culture medium consisted of infusions of hay and grass, but no particular care was taken to keep the medium the same from day to day....In some cases the liquid was used as soon after it had been boiled as it had attained the room temperature, in other cases it was allowed to stand in a stoppered flask for twenty-four hours or more. But from February 1908 to the present time a more varied culture medium has been used...materials for the infusions were taken practically at random, from laboratory aquaria, hay infusions, ponds, etc. The infusions were thoroughly boiled to prevent the contamination of the pure lines of the pedigree culture by wild individuals."

Since boiling does not diminish the stimulating effect of bacterised cultures and many of the infusions described by Woodruff must have been heavily infected with bacteria before boiling, intermittent stimulation of reproductive capacity through the presence of "X-substance" in the culture media must evidently have occurred.

For over 200 generations my laboratory cultures of *Enchelys*, propagated asexually, have shown no consistent diminution of reproductive capacity. It is, however, very easy to cause a rapid loss of reproductive capacity by repeatedly subculturing from old and "starved" cultures into sterile hay infusions. In two or three generations a high mortality prevails among isolated individuals, and the reproductive rate of those which survive is very low. Immediate restoration of viability and reproductive rate is achieved by cultivation into bacterised infusions, or, more slowly, by repeated propagation from young cultures. It is of very great interest in view of results which are about to be described, that whereas isolated individuals from cultures which have repeatedly been propagated from old parent cultures almost invariably die, "mass-cultures" propagated by introducing 20 or 30 individuals simultaneously into the infusion invariably survive. Either some of the individuals retain sufficient viability to populate the culture, or the contiguity of numerous cells promotes the viability and multiplication-rate of each of them. In view of what follows the latter must be regarded as a probable explanation.

It has been observed by Jennings, who employs freshly-extracted and boiled hay infusions and carefully sterilises all of the glassware employed, that "there are some stocks (of *Paramoecium*) which will not stand long continued cultivation on slides at all, although they live perfectly in mass-culture." In my experience it makes no difference whether the culture inhabits a drop of infusion contained in a depression in a slide, or the same fluid contained in some larger vessel, provided only that a number of individuals are simultaneously introduced into the new culture liquid.

The Time-relations of Multiplication in Cultures of isolated Infusoria.

It has been shown in a number of investigations [Robertson, 1908, 1913, 1915, 1916; Ostwald, 1908; McKendrick, 1910] that the time-relations of growth, whether in bacterial cultures or in the tissues of the higher animals, are similar to those which obtain in chemical reactions which produce their own catalysors. The bacterial count in cultures of bacteria, or the total weight of the body in higher animals or plants, increases at first very slowly, but as the cells increase in number the velocity of multiplication increases proportionately until it attains a maximum, from which it falls off to the condition of stasis which marks the end of multiplication in the culture or of growth in the animal. In the *Vertebrata* periods or "cycles" of growth alternate with periods of relative stasis, but each of the growth-cycles, such as for instance that which initiates *in utero* and culminates towards the end of the first year of extra-uterine life in man [Robertson, 1916], reproduces with truly surprising exactitude the quantitative characteristics of an autocatalysed chemical reaction, the weight of the animal or infant taking the place of the mass or concentration of the product in the chemical reaction-formula.

Precisely similar quantitative relationships are exhibited during the growth of a culture of infusoria. There is a more or less prolonged "lag-period" during which multiplication is very slow or apparently arrested. This is succeeded by a progressively increasing rapidity of multiplication which, after passing through a maximum, subsequently sinks and ultimately falls to zero level. Hence multiplication is always much less rapid during the first 24 hours after isolation than it is during the second 24 hours, even in bacterised infusions. The following are illustrative results among a very large number recorded during the progress of these experiments:

Normal Hay Infusions.

	Individuals in subculture after		Number of divisions during	
Culture number	24 hours	48 hours	24 hours	48 hours
51 A	2	30	1	5
51 B	1	10	0	3
215 A	1	26	0	4·5
217 A	2	61	1	6

The same relationships are exhibited when shorter time-intervals are employed in more rapidly multiplying cultures. The curves in Fig. 2 illustrate some such instances, and it will be seen that they correspond closely to the earlier or ascending part of the curve which displays the relationship between the mass of the product and the time in an autocatalysed chemical reaction. The positions of points on the further continuation of the curve are not easy to determine on account of the difficulty of counting so many actively moving individuals, and I have as yet made no attempts in this direction. The data set forth above, however, taken in conjunction with the readily ascertainable fact that reproduction ceases altogether after the population of the culture

has become very numerous, show that the reproduction of infusoria displays the general autocatalytic characteristics which are encountered in other types of growth.

In the case of infusorial cultures, however, a special complication is introduced by the fact that the food of the organisms is supplied by bacteria which are simultaneously multiplying in the culture. The possibility is thus suggested that the autocatalytic character of the multiplication of infusoria may merely be the reflection of the autocatalytic relations governing the multiplication of bacteria, which have been evaluated by McKendrick [1910]. Part of the effect must doubtless be attributable to this source, but that it is not entirely attributable to the autocatalysed multiplication of food-materials is shown by the fact that the auto-acceleration is displayed to a pronounced degree in bacterised infusions. The "lag-period" is somewhat less in duration, but still very perceptible, and the reproductive rate, although more rapid in fresh hay infusions, undergoes progressive acceleration with the lapse of time and increase of the population. The following is an illustrative experiment, which is also depicted in curves 237 A and 237 B in Fig. 2.

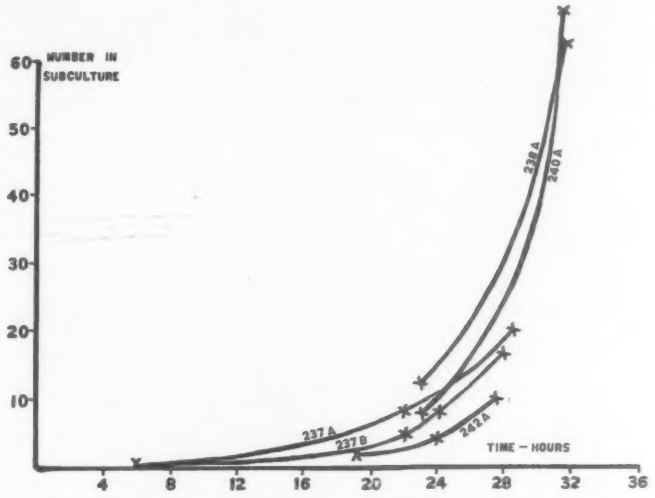

Fig. 2. Showing the auto-acceleration of multiplication-rate in cultures arising from single isolated infusoria.

Bacterised Hay Infusion, unfiltered.

Culture number	Number of divisions which had occurred after 6 hours	22 hours	24 hours	28 hours
237 A	0	3	3	4·5
237 B	0	2	3	4·0

Thus in the first six hours after isolation, and in the presence of abundance of food, no divisions occurred, but in the six hours between 22 and 28 hours after isolation one-and-a-half

divisions occurred in the culture 237 A, yielding a total population of 20 individuals, and two divisions occurred in the culture 237 B, yielding a total population of 16 individuals. In the first 22 hours divisions occurred at the rate of one in seven hours in culture 237 A, and of one in 11 hours in culture 237 B, but in the succeeding six hours divisions occurred at the rate of one in four hours in culture 237 A and one in three hours in culture 237 B. Similar phenomena were exhibited, without any exception, by all of the cultures in which counts were made at several successive intervals of time.

The multiplication of infusoria, therefore, partakes of the autocatalytic character which has been found to distinguish multiplication of cells and growth in all of the various forms of life which have hitherto been investigated from this point of view. It must therefore be regarded as the expression of a fundamental property of protoplasm. Obviously it depends upon the contiguity of the cells to one another in a limited space and establishes the existence of a mutual influence upon one another's multiplication-rate which will be discussed more fully in the succeeding article.

The Influence of Temperature upon the Viability of Cultures and the Protective Effect of Mutual Contiguity.

The temperature of 30° C. prevents subcultures from multiplying, and the isolated individual almost invariably dies. The following are illustrative experiments:

Normal Hay Infusion.

Culture number	Age of parent culture	Temperature	Individuals in 24 hours
112 A	3 days	30°	0
117 A	3 „	32°·8	0
117 B	3 „	32°·8	0
125 A	3 „	29°·5	0
125 B	3 „	29°·5	8
152 A	2 „	30°	0
152 B	2 „	30°	4

Control cultures from the same parent cultures and kept at lower temperatures displayed normal reproductive capacity. Exactly the same phenomena were exhibited in bacterised infusions:

Bacterised Hay Infusion, unfiltered.

Culture number	Age of parent culture	Temperature	Individuals in 24 hours
153 A	2 days	30°	0
153 B	2 „	30°	0
156 A	4 „	30°·5	0
156 B	4 „	30°·5	0
157 A	2 „	30°·5	0
157 B	2 „	30°·5	0

Hence paucity or abundance of food does not influence the result. That true thermal death has occurred and not merely encystment is shown by the fact that these cultures never recover or become inhabited by infusoria after removal to a lower temperature.

This high degree of thermal sensitivity is very remarkable because shade temperatures above 30° C. are very usual during the summer months in South Australia, so that these organisms must frequently be under necessity of withstanding temperatures which, under the laboratory conditions specified, are invariably fatal. It was at first thought that the high degree of thermal sensitivity might be due to repeated culturing for very many generations under laboratory conditions, and to test this point "wild" individuals were obtained and cultured under identical conditions. All of the cultures exposed to temperatures above 30° C. died, while cultures into the same infusion at lower temperatures showed reproductive capacity similar to that of the laboratory strains.

It is possible that under conditions of simultaneous heat and desiccation viability at high temperatures may be increased by the assumption of a condition of arrested metabolism, *i.e.* encystment. But another possible means whereby "wild" infusoria of this species may be able to survive summer conditions is indicated by the fact that well-populated cultures are able to withstand much higher temperatures than isolated individuals. During the summer months well populated slide-cultures of the laboratory strains have successfully withstood exposure to room temperatures of 33° to 34° C. for from five to seven days in succession without death or any apparent injury or abnormality. Moreover subcultures propagated on slides by the introduction of 20 or 30 individuals into fresh hay infusion invariably survive and multiply at these temperatures, whereas single individuals, isolated from the same parent culture and habituated to the high temperature, invariably perish.

This result cannot be attributed to the introduction of greater numbers of bacteria together with the greater numbers of infusoria into the fresh hay infusion, because the viability of isolated individuals at high temperatures is not in any way increased by previous bacterisation of the infusion. Again we are led to infer the existence of a mutual influence, arising from the contiguity of cells, which in this instance is protective, enabling the organisms to withstand some adverse condition arising out of the high temperature of the environment which is fatal to isolated individuals.

The nature of the adverse agency at high temperatures is not yet ascertained. It is conceivable that it is a direct action of the temperature upon processes occurring in the organism itself, leading to the formation of toxic products of metabolism, to over-rapid metabolism, or destruction of essential enzymes, or, on the other hand, that the action of high temperatures is indirect, arising from the development of an adverse type of bacteria, or of an excess of certain bacterial products. The experimental evidence which is as yet available does not suffice to decide between these alternatives. But from whatever source the adverse effects of temperatures above 30° C. may arise, the protective effect of mutual contiguity of the organism remains unquestionable.

SUMMARY.

1. The rate of multiplication of single infusoria (*Enchelys farcimen* Ehr.) isolated from parent cultures of various ages falls off, at first very rapidly and later more slowly, with increasing age of the parent culture.

2. This loss of reproductive capacity has been shown not to be attributable to the accumulation of any retarding substances in old cultures, but to a period of existence at a low nutritive level.

3. Bacterisation of the hay infusions employed as culture media, by preliminary standing at room temperatures for from 24 to 72 hours, remarkably stimulates the reproductive capacity of the isolated organisms, and it delays, but does not prevent the loss of reproductive capacity which occurs with advancing age of the parent culture.

4. Heating the bacterised infusion to boiling-point, or even for 90 minutes on a boiling water-bath just before its employment, does not diminish the multiplication-rate of individuals isolated into it.

5. Although the final number of infusoria produced in a given culture is sooner or later determined by the number of bacteria available for food, yet the initial accelerative effect of bacterised infusions upon reproductive rate is not diminished by removal of the bacteria by filtration through a Berkefeld filter.

6. The accelerative effect of the bacterised infusion is not, therefore, dependent upon the presence of food, *i.e.* of bacteria, living or dead, but upon the presence of a soluble, non-volatile, thermostable product arising from the inhabitation of the medium by bacteria. It is proposed to distinguish this substance by the provisional designation of "X-substance."

7. The multiplication-rate undergoes a progressive increase with each successive division during the early stages of the development of a culture. Hence the multiplication of infusoria partakes of the autocatalytic character which has previously been shown to distinguish other types of growth, in higher plants and animals as well as in cultures of unicellular organisms.

8. This autocatalysis of multiplication is due to the contiguity of the cells to one another in a limited space, and is not merely the expression of the increasing abundance of foodstuffs due to the simultaneous multiplication of bacteria in the culture.

9. Temperatures above 30° C. prevent subcultures of single individuals of *Enchelys farcimen* from multiplying, and the isolated individual at this temperature almost invariably dies.

10. Cultures initially containing a large number of individuals, however, will multiply and survive continued exposure to considerably higher temperatures. Individuals isolated from such cultures and kept at the same temperature invariably die. Hence the contiguity of many organisms affords mutual protection against the adverse effects of high temperatures.

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LXXIV. EXPERIMENTAL STUDIES ON CELLULAR MULTIPLICATION.

II. THE INFLUENCE OF MUTUAL CONTIGUITY UPON REPRODUCTIVE RATE AND THE PART PLAYED THEREIN BY THE "X-SUBSTANCE" IN BACTERISED INFUSIONS WHICH STIMULATES THE MULTIPLICATION OF INFUSORIA.

By THORBURN BRAILSFORD ROBERTSON.

From the Department of Physiology and Biochemistry of the University of Adelaide, South Australia.

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The Reproductive Rate in Cultures of two Individuals.

When two individuals from the same young parent culture of *Enchelys farcimen* are separately isolated and re-isolated, in the manner described in the preceding article, and are then introduced together into the same large drop (0·08 cc.) of hay infusion, the result is usually that not twice but from four to six times as many individuals are produced in 24 hours as in cultures of but a single individual. The following are illustrative of numerous experiments:

Normal Hay Infusion; Parent Culture 24 hours old.

Number of culture	Number of parent culture	Number of individuals originally introduced	Number after 24 hours	Ratio
15 B	14 B	1	12	
16 B	„	2	60	1 : 5·0
19 B	17 B	1	16	
21 B	„	2	90	1 : 5·6
36 A	35 A	1	60	
37 A	„	2	260	1 : 4·3
64 B	63 B	1	24	
65 B	„	2	102	1 : 4·2

Normal Hay Infusion; Parent Culture 48 hours old.

41 A	38 A	1	4	
42 A	„	2	30	1 : 7·5
41 B	38 B	1	5	
42 B	„	2	22	1 : 4·4
47 A	45 A	1	2	
48 A	„	2	8	1 : 4·0
62 A	61 A	1	8	
63 A	„	2	20	1 : 2·5
62 B	61 B	1	7	
63 B	„	2	20	1 : 2·9
188 A	184 A	1	2	
189 A	„	2	8	1 : 4·0
188 B	184 B	1	2	
189 B	„	2	11	1 : 5·5

Normal Hay Infusion; Parent Culture 72 hours old.

Number of culture	Number of parent culture	Number of individuals originally introduced	Number after 24 hours	Ratio
23 A	19 A	1	1	
24 A	„	2	6	1 : 6
23 B	19 B	1	2	
24 B	„	2	6	1 : 3
191 A	184 A	1	2	
192 A	„	2	6	1 : 3
191 B	184 B	1	1	
192 B	„	2	6	1 : 6

Pairs of individuals isolated from parent cultures older than three days either do not exhibit this phenomenon at all or only irregularly. The following is an illustrative experiment:

Normal Hay Infusion; Parent Culture in each instance Number 184.

Number of culture	Age of parent culture	Number of individuals	Individuals after 24 hours	Ratio
188 A	2 days	1	2	
189 A	2 „	2	8	1 : 4
191 A	3 „	1	2	
192 A	3 „	2	6	1 : 3
207 A	8 „	1	8	
208 A	8 „	2	16	1 : 2
188 B	2 „	1	2	
189 B	2 „	2	11	1 : 5·5
191 B	3 „	1	1	
192 B	3 „	2	6	1 : 6
207 B	8 „	1	6	
208 B	8 „	2	16	1 : 2·7

An individual isolated from an old culture and placed in the same drop of culture medium has no effect upon the reproductive rate of an individual isolated from a younger culture. The following experiment illustrates this:

Normal Hay Infusion; Parent Culture 24 hours old.

Number of culture	Number of individuals originally introduced	Number of individuals produced after 24 hours
55 B	1	14
56 B	2	48
57 B	1 from above culture plus 1 from culture 5 days old	17

The possibility that the mutually accelerative effect of two individuals is due to their conjugation may readily be dismissed. In the first place repeated observation of such cultures hour by hour up to the beginning of multiplication by fission has never revealed the occurrence of conjugation. In the second place, as Jennings has convincingly shown [Jennings, 1913], the actual effect of conjugation upon infusoria is not to accelerate but very greatly to retard their reproductive rate. Finally the only conjugants which I have ever obtained in this species have been among individuals recently washed in distilled

water, and individuals which have been so treated, as we shall see, actually fail to display the mutual accelerative effect.

At first it appeared likely that the phenomenon was simply due to the more effective seeding of the fresh hay infusion with bacteria by contamination with the parent culture fluid associated with two individuals instead of one, the available food supply being thus increased in the cultures containing two individuals. Hence many experiments were carried out upon the effects of repeated washing of the infusoria upon their ability thus mutually to influence each other's reproductive rate. The results distinctly supported the above explanation, because it was found that repeated washing in faintly alkaline water (2 cc. of $N/10$ Na_2CO_3 per 100 cc. of distilled water) completely abolished the effect. The following is one of many similar experiments:

Normal Hay Infusion; Parent Cultures Number 223 (A *and* B).

The washed individuals were suspended five successive times in 50 volumes of the faintly alkaline distilled water and recovered each time by gentle centrifugalisation which did not deform or injure them in any way.

Number of culture	Treatment of isolated individuals	Number of individuals	Individuals after 24 hours
224 A	Unwashed	1	8
225 A	Unwashed	2	24
226 A	Washed	1	4
227 A	Washed	2	5

This explanation completely broke down, however, when it was discovered that the mutual acceleration of reproductive rate by two associated individuals occurs, frequently in an even more pronounced degree, in previously bacterised infusions. The added infection carried into a bacterised hay infusion in association with the infusoria, the parent culture fluid having been diluted at least 400 times and generally many multiples of this by the new infusion, must be insignificant in comparison with the overwhelming bacterial population already present. Furthermore in bacterised infusions washing the infusoria failed to abolish or even to modify the effect. The following experiment shows this.

Normal hay infusion was bacterised by admixture with a small proportion of infusion which had been prepared 24 hours earlier and in hot weather. The parent culture was 3 days old.

Number of culture	Number of individuals	Treatment of individuals	Individuals after 24 hours
202 A	1	Unwashed	31
203 A	2	Unwashed	98
204 A	2	Washed	89

Quite convincing evidence that the accelerative effect of the second individual is not directly due to the heavier infection with associated bacteria was also afforded in another way. In the preceding article it has been pointed out that the thermal sensitivity of *Enchelys* is very great, temperatures above 35° C. being usually, and temperatures above 40° C. invariably fatal even for cultures containing many individuals. The hay bacillus is, of course, not

adversely affected by brief exposures to such temperatures. Accordingly two individuals were separately isolated and re-isolated from the same parent culture. One was killed by heating the slide by placing it for three minutes in an incubator heated to 50° C. The two small drops, one containing the living and the other the killed infusorian, were then mixed, diluted with 0·08 cc. of fresh hay infusion, and allowed to stand at room temperature as usual. Control experiments with single individuals from the same parent culture were simultaneously performed. Out of several such experiments affording similar results the following is one:

Normal Hay Infusion; Parent Culture 2 days old.

Number of culture	Individuals introduced	Number after 24 hours
242 A	1	8
243 A	2	31
244 A	1 living + 1 killed	8
242 B	1	10
243 B	2	34
244 B	1 living + 1 killed	9

Had the stimulative effect of the second individual in cultures 243 A and B been due to associated bacteria, then cultures 244 A and B should have contained at least 16 individuals each.

The question then arises why washing the infusoria cultured into fresh normal hay infusion abolishes the mutually accelerative effect. That the effect of washing is not upon the infusoria themselves, *i.e.* due to direct injury to their reproductive capacity, is shown by the fact that the mutual acceleration occurs if the washed infusoria are cultured into bacterised infusions. Evidently the loss of ability mutually to accelerate reproduction is due to the removal by washing of some associated organisms or substances which are present in abundance in bacterised infusion, although, as we have seen, the acceleration itself is not directly due to the contamination. In other words, as more extended experimentation with relatively sterile infusions now showed, *the mutual acceleration is never exhibited in the absence of a certain minimum of bacteria or their products*, although the bacteria themselves, in the absence of the second infusorian, are not able to bring about an equal acceleration of reproductive rate.

Before these facts had been ascertained, and the great importance of the bacterial population of the infusions had been established, it was not considered essential to sterilise every container used in the preparation of the hay infusions. In particular the small wide-mouthed stoppered bottle in which the infusion was placed after it was cooled and immediately before use was usually merely washed out with water between successive experiments. The occurrence of the mutually accelerative effect in "Normal Hay Infusions," so frequently displayed in the experiments cited above, was ultimately traced to this source. If the infusion is thoroughly sterile, and the infusoria are re-isolated several times, a negative result, *i.e.* absence of mutually accelerative effect, is invariably

obtained in cool weather. In hot weather, when bacteria multiply rapidly in the infusions, a negative result is less readily obtained, and in any case if the cultures be exposed to the air without a cover for some time, or any container used in preparing the medium has not been either boiled or else rinsed with ether, a positive result is usually obtained.

In some instances negative results are obtained in heavily bacterised infusions. It is not certain that this may not be due to the presence of an unfavourable type of bacterial population, but the impression has been gained, although not as yet fully confirmed, that there exists an optimum density of bacterial population for the exhibition of the effect, above which the effect dwindles and ultimately disappears.

The ability to cause mutual acceleration of reproductive rate is, as we have seen, lost with advancing age of the parent culture. It may be restored by re-culturing and sub-culturing from the young cultures. It is also gained with the growth of the individual infusoria, for a dividing individual, or two individuals which have only just completed division, when isolated into fresh hay infusion, behave like single individuals and not like pairs of individuals. The following are illustrative of a number of experiments:

Normal Hay Infusion; Parent Culture 24 hours old.

Number of culture	Individuals introduced	Number after 24 hours
55 A	1	14
56 A	2	56
57 A	1 (dividing)	16

Normal Hay Infusion; Parent Culture 2 days old.

Number of culture	Individuals introduced	Number after 24 hours
188 A	1	2
189 A	2	8
190 A	1 (dividing)	2

These experiments clearly reveal the origin of the increase of reproductive rate with population which occurs in cultures of infusoria. Doubtless the individuals arising from the division of the single isolated cell, when mature, influence each other's division-rate in just the same way as any two individuals isolated from the same parent culture. The next division multiplies the effect, and hence arise the peculiar time-relations of multiplication which have been dwelt upon in the preceding article. Since this autocatalytic increase of reproductive rate is characteristic of all congeries of living cells, we may presume that in all cases it arises out of an analogous mutual influence of contiguity. No effect of mutual contiguity can readily be imagined which is not transmitted from one cell to another, across the intervening fluid medium, through the agency of a soluble substance secreted from the cells. The existence of autogenous catalysors of cellular multiplication, therefore, must necessarily be inferred, at least in the case of infusoria, and the autocatalytic theory of growth, hitherto an hypothesis founded upon and supported by indirect evidence, has now, in a particular instance, been demonstrated to be true.

The Nature of the Substance in Bacterised Infusions which induces the Mutually Accelerative Effect.

The mutually accelerative effect of pairs of infusoria upon their reproductive rate occurs, as we have seen, only in media which contain or have contained bacteria. The actual presence of the bacteria is unnecessary, however, provided the soluble products of their metabolism are present. Filtration of the bacterised infusions through a Berkefeld filter does not abolish or even in the least diminish the accelerative effect. The following experiments show this:

Normal hay infusion was made up with every precaution to prevent infection by bacteria. A portion of this was mixed with an equal volume of an infusion which had been allowed to stand for two days at room temperature and then filtered through a Berkefeld filter. Microscopic examination showed the filtrate to be free from bacteria. The parent culture employed was three days old.

Number of culture	Culture medium	Individuals introduced	Number after 24 hours	Ratio
249 A	Normal hay infusion	1	8	
250 A	ditto	2	13	1 : 1·6
251 A	Same plus equal volume of filtered, bacterised infusion	1	14	
252 A	ditto	2	38	1 : 2·7
249 B	Normal hay infusion	1	8	
250 B	ditto	2	16	1 : 2·0
251 B	Same plus equal volume of filtered, bacterised infusion	1	10	
252 B	ditto	2	85	1 : 8·5

The effect is also obtained in pure filtered bacterised infusion, unmixed with fresh infusion, but as in this case neither bacteria nor bacterial spores are present reproduction necessarily comes to a standstill after a few divisions for lack of food. These organisms display an extremely high reproductive rate when transferred to fresh hay infusion.

The effect is not diminished by boiling the bacterised infusion, either before or after passage through a Berkefeld filter, nor is it appreciably diminished by heating the infusion for 90 minutes on a boiling water-bath. The substance which enables the effect to occur cannot be conducted by distillation from a bacterised infusion into a fresh hay infusion. In other words it resembles exactly the substance which, as shown in the preceding article, stimulates the reproductive rate of single, isolated individuals. There can be little doubt that the stimulation of reproductive rate by the "X-substance" in bacterised infusions is, in reality, not directly due to the substance itself, but to a substance produced from it, or through its agency, by the infusoria. Two infusoria produce a greater quantity than one, and the reproductive rate of each is proportionate to the concentration of this product in the medium.

Discussion of the Results.

That in media which are most favourable to rapid multiplication, contiguous cells are able to catalyse each other's reproductive rate, has been demonstrated in these experiments by direct observation of the effect. The existence of such an effect has previously been inferred from the quantitative time-relations of growth in the higher plants and animals and of multiplication in cultures of unicellular organisms, but the direct demonstration of its occurrence has also brought to light the fact that, at least in infusorial cultures, the effect is entirely dependent upon the presence of a soluble substance in the culture medium, which is produced by bacteria, and which is requisite for the manufacture of the autocatalyst by the infusoria themselves. There is, of course, no evidence as yet to show that the autocatalytic character of growth in higher animals is similarly dependent upon the presence, in the nutrient media bathing the cells, of any analogous substance, but the possibility is certainly indicated that a food-accessory may exist which is not of itself essential to growth, but which constitutes a raw material for the manufacture of growth-catalysors by the animal cells.

The "X-substance" which enables the production of growth-catalysors by infusoria is not identical with the water-soluble Vitamin B, because it resists boiling [see Robertson, 1921, p. 604]. Whether or not it is related to the fat-soluble group of accessories, or to any hitherto undiscovered accessory, remains to be ascertained.

On the other hand, given the presence of the X-substance, or raw-material, the ability of the infusoria to utilise it for the manufacture of the catalysor plainly varies with their physiological state. It diminishes during periods of starvation, or at any rate paucity of foodstuffs, and recovery is a matter of some time. The possibility may be suggested that in a densely inhabited culture, containing but few bacteria relatively to infusoria, the entire amount of catalysor produced is needed by the infusoria themselves, and remains "locked up" in their protoplasm, no excess being available for discharge into the surrounding medium. On restoration to a medium containing excess of X-substance, some time is required before a sufficient amount of catalysor can be synthesised to supply the infusoria with a sufficient content to leave a surplus for secretion into the surrounding medium. Individuals isolated from young cultures, on the other hand, start discharging excess of catalysor directly after their isolation into a medium containing the necessary supply of X-substance. The rate of production of the catalysor appears, from the autocatalytic growth-formula, to be directly proportionate to the mass of protoplasm, and this is probably the reason why a dividing infusorian, containing as it does very little greater mass of protoplasm than any other mature individual in the same culture, is able to synthesise no more of the catalysor in a given time than a single undividing individual.

SUMMARY.

1. When two individuals from the same parent culture are introduced together into the same drop of culture medium, not twice, but from four to six or eight times as many individuals are produced in 24 hours as in cultures which initially contained but a single individual.

2. The mutually accelerative effect of pairs of infusoria upon reproductive rate has been demonstrated not to be due to the more effective seeding of the culture medium by the bacteria contaminating two individuals, since it occurs equally in media which are densely inhabited by bacteria. It is also not attributable to conjugation of the associated organisms.

3. The autocatalytic theory of growth, hitherto an hypothesis founded upon and supported by indirect evidence, has thus, in a particular instance, been demonstrated to be true.

4. The accelerative effect of mutual contiguity is never exhibited excepting in the presence of soluble products of bacterial metabolism, but bacteria themselves are not essential.

5. The product which enables the effect to occur is non-volatile and thermostable. It appears to be identical with the X-substance in bacterised infusions which accelerates the multiplication of isolated single individuals.

6. The X-substance is therefore not itself an accelerator of multiplication, but is converted by animal cells into an accelerative agent, or else enables animal cells to produce an accelerative agent from other sources.

7. If the autocatalytic character of growth in the higher animals originates in a similar phenomenon, then the possibility is indicated of the existence of an accessory foodstuff which is essential, not by reason of its own direct action upon growth, but because it enables the production of autogenous catalysors of cell-multiplication by the animal cells themselves.

8. The relationship of the X-substance to the hitherto known classes of accessory foodstuffs has not yet been elucidated. It is distinguishable from the water-soluble Vitamin B, however, by its thermostability.

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LXXV. THE PENETRATION OF ELECTROLYTES INTO GELS.

III. THE INFLUENCE OF THE CONCENTRATION OF THE GEL ON THE COEFFICIENT OF DIFFUSION OF SODIUM CHLORIDE.

By WALTER STILES and GILBERT SMITHSON ADAIR.

From the Department of Botany, University College, Reading, and King's College, Cambridge.

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Introduction.

The influence of the concentration of a gel on the rate of diffusion of salts through it is a question on which the most divergent opinions have been expressed. Whereas de Vries [1884], Hagenbach [1898], Bechhold and Ziegler [1906], and Öholm [1905, 1913] concluded that the presence of gelatin had a retarding effect on the rate of diffusion, the determinations of Morse and Pierce [1903] suggest the contrary, while Henri and Calugareanu [1903] came to the conclusion that if the gelatin had not set to a gel the rate of diffusion of organic dyes was the same as in water, but that if the gelatin had set the rate of diffusion was slower. Voigtländer [1889] concluded that in agar-agar gels any differences observed in the coefficients of diffusion of a number of electrolytes in gels of different concentrations were within the limits of experimental error. Incidental observations recorded in previous papers of this series support the conclusion of the comparatively small influence of the gel on diffusion in agar-agar gels. [Stiles, 1920; Adair, 1920.]

Since recent investigations on the structure of protoplasm all point to the conclusion that the living materials of cells may in some cases exist in the gel condition, it is of physiological as well as of chemical interest to obtain exact information with regard to the influence of the gel on the diffusion of electrolytes and other substances of importance in the life of the organism.

Method of Experiment.

Most of the determinations already made of coefficients of diffusion, have been made by the chemical analysis of different layers of the medium through

which diffusion is allowed to take place. While the indicator method used for the determinations described in this paper has found considerable employment in the past for observing the penetration of electrolytes, definite measurements of the coefficient of diffusion by its means have only become possible by the application of Fourier's equations to this special case as worked out in the previous contribution in this series of studies [Adair, 1920]. The advantages of the indicator method are described in that paper and need no repetition here.

Measurements were made with both gelatin and agar-agar gels containing silver nitrate as indicator. As experimental salt sodium chloride was employed as in the earlier work previously described. The concentrations of gel, indicator and diffusing salt employed are recorded in the section dealing with the experimental results. It speaks for the validity of the method that the values obtained with the stronger silver nitrate concentration in the gel are practically identical with those obtained with the weaker strength of silver nitrate.

The diffusion was allowed to proceed from a large volume of salt solution into inverted narrow tubes containing the gel; care has to be taken in the performance of the experiments that evaporation does not take place either from the surface of the liquid or from the upper end of the gel. The formulae necessary for the calculation are to be found in the second paper [Adair, 1920].

The experiments were carried out in thermostats maintained at 0° and 20° in the case of gelatin and at 0°, 20° and 40° in the case of agar-agar. The last named temperature is too high for experiments with gelatin. During the time of the experiment the temperature of the bath rarely changed more than 1/10 of a Centigrade degree, and generally not nearly so much.

Experimental Results.

1. *Gelatin.*

Experiments were conducted on two samples of gelatin. In both cases the gelatin on analysis proved to contain chloride, although in the first sample the quantity of this was much greater than in the second sample. As the presence of any substance that will remove the silver nitrate from solution, apart from the experimental diffusing salt, will vitiate the results of the subsequent calculations, it was necessary to remove the chloride by successive washing with distilled water, until this gave no precipitate with silver nitrate after it had remained for some time in contact with the gelatin. This process was carried out in all cases.

The experimental data are given in the tables below. Each determination is the mean of six or more readings, but it was not considered necessary to publish the results for each individual tube; in order to show the degree of variation between different tubes treated in the same way the results for the first set of readings in three experiments (Nos. 3–5) are given in full in Table I.

Table I. Penetration of sodium chloride from N solution into gelatin gels containing 0·01 N silver nitrate at 0° C.

Concentration of gel	4 %	8 %	16 %
Time of diffusion	22·63 hours	22·525 hours	22·71 hours
Penetration in cm.	2·70	2·50	2·32
	2·71	2·53	2·28
	2·70	2·60	2·28
	2·71	2·53	2·30
	2·70	2·58	2·28
	2·70	2·54	2·28
	2·72	2·59	2·31
	2·70	2·52	2·31
	2·70	2·55	—
	2·72	2·59	—
Mean penetration in cm.	2·706	2·553	2·295
Probable error of mean...	0·002	0·007	0·004
Coefficient of diffusion in c.g.s. units...	$0·675 \times 10^{-5}$	$0·604 \times 10^{-5}$	$0·483 \times 10^{-5}$

The numbers given above will give sufficient idea of the degree of concordance between different tubes in the same experiment. Similar agreement was obtained in all the results recorded in Table II, p. 623.

2. *Agar-agar.*

In determining coefficients of diffusion in gels of agar-agar the experimental difficulties are greater than in the case of gelatin. One fruitful source of error is evaporation from the upper end of the tube of gel especially in the case of low concentrations and at higher temperatures. In gels of high concentrations bubbles in the gel are often difficult to eliminate and the presence of these may lead to incorrect results. In the third place, any heating of the gel after addition of silver nitrate leads to a darkening of the gel. If this is to be interpreted as due to decomposition of some of the silver nitrate, its incidence will lead to values of the coefficient of diffusion which are higher than the true ones. The results given below are of experiments free from these sources of error. Experiments were conducted at three temperatures—0°, 20° and 40°. The values obtained for the coefficient of diffusion at these three temperatures in gels containing respectively 0·5, 1, 2 and 4 per cent. of agar-agar are summarised in Tables III and IV.

Table III. Coefficient of diffusion of sodium chloride at different temperatures in agar-agar gels of different concentrations. (Shred agar-agar.)

Concentration of agar-agar in %	Coefficient of diffusion in c.g.s. units $\times 10^5$		
	0·0°	20·1°	40·2°
0·5	0·781	1·386	—
1·0	0·765	1·357	2·143
2·0	0·738	1·328	2·084
4·0	0·711	1·290	2·061

Table II. Penetration of sodium chloride from *N* solution into gelatin gels with silver nitrate as indicator. (Concentration of gelatin in percentage by weight, time in hours, penetration in centimetres of precipitate. Diffusion coefficient, *k*, in c.g.s. units $\times 10^5$.)

Experiment	Concentration of gelatin. %	Concentration of $AgNO_3$	Temperature ° C.	Time	Penetration	*k*
1	5	0·01 *N*	0	20·65	2·575	0·670
				43·58	3·735	0·668
2	15	,,	,,	20·28	2·237	0·515
				43·15	3·258	0·513
3	4	,,	,,	22·63	2·706	0·675
				46·57	3·903	0·683
4	8	,,	,,	22·525	2·553	0·604
				46·64	3·705	0·614
5	16	,,	,,	22·71	2·295	0·483
				46·70	3·312	0·490
6	2	0·005 *N*	,,	17·775	2·6525	0·697
				41·57	4·1025	0·713
7	4	,,	,,	17·85	2·630	0·6835
				41·625	4·034	0·688
8	8	,,	,,	17·93	2·533	0·630
				41·69	3·880	0·636
9	16	,,	,,	17·99	2·289	0·513
				41·74	3·487	0·513
10	2	,,	,,	17·905	2·703	0·719
11	4	,,	,,	17·98	2·605	0·664
12	8	,,	,,	18·075	2·530	0·624
13	16	,,	,,	18·15	2·342	0·532
14	5	0·01 *N*	20	20·37	3·435	1·209
				43·43	5·040	1·221
15	15	,,	,,	20·00	3·043	0·967
				43·03	4·490	0·978
16	4	,,	,,	22·18	3·651	1·254
				48·075	5·385	1·260
17	8	,,	,,	22·29	3·507	1·152
				48·15	5·120	1·137
18	16	,,	,,	22·39	3·164	0·934
				48·22	4·666	0·943
19	2	0·005 *N*	,,	22·53	4·049	1·281
				46·32	5·816	1·285
20	4	,,	,,	22·61	4·0025	1·248
				46·40	5·710	1·237
21	8	,,	,,	22·675	3·864	1·160
				46·48	5·481	1·139
22	16	,,	,,	22·76	3·500	0·950
				46·55	4·974	0·936
23	2	,,	20·3	17·31	3·577	1·302
				41·99	5·607	1·318
24	4	,,	,,	17·41	3·504	1·243
				42·04	5·456	1·247
25	8	,,	,,	17·51	3·334	1·118
				41·93	5·209	1·140
26	16	,,	,,	17·62	3·150	0·9915
				42·11	4·899	1·003

Table IV. Coefficient of diffusion of sodium chloride at different temperatures in agar-agar gels of different concentrations. (Powdered agar-agar.)

Concentration of agar-agar in %	Coefficient of diffusion in c.g.s. units × 10^5		
	0·0°	20·0°	40·0°
0·5	0·782	1·382	2·205
1·0	0·761	1·371	2·126

The agreement between these two sets of results is very good; there is evidently no difference between shred and powdered agar-agar in regard to the diffusivity of sodium chloride through gels made from these two kinds of agar-agar.

Taken by themselves these results form a perfectly regular series indicating a progressive reduction in the diffusivity with increasing concentration of the gel. But when the values for diffusivity in a gel of concentration 0 are obtained by extrapolation they are found to differ both from those obtained in a similar way by the use of gelatin gels and those obtained by extrapolation from Öholm's determinations for the diffusivity of sodium chloride. Some discussion of the results is obviously necessary.

Discussion of Experimental Results.

In the preceding section of this paper we have recorded the values obtained by the indicator method for the coefficient of diffusion in gels of gelatin and agar-agar of various concentrations. From these data the following conclusions can be drawn:

1. In gelatin the relation between the coefficient of diffusion (k) and the concentration of gelatin (c) can be expressed by the equation

$$k = a\,(1 - bc)$$

where a and b are arbitrary constants, and when $c > 2\,\%$. The values of a and b calculated from the data presented are as follows:

At 0° for a gel containing 0·01 N silver nitrate, $a = 0{\cdot}74$; $b = 0{\cdot}0207$.
„ 0° „ „ 0·005 N „ „ $a = 0{\cdot}734$; $b = 0{\cdot}018$.
20° „ „ 0·01 N „ „ $a = 1{\cdot}35$; $b = 0{\cdot}0185$.
„ 20° „ „ 0·005 N „ „ $a = 1{\cdot}35$; $b = 0{\cdot}0185$.
20·3° „ „ 0·005 N „ „ $a = 1{\cdot}36$; $b = 0{\cdot}017$.

2. The results for agar-agar indicate that the coefficient of diffusion does not decrease uniformly with increase in the concentration of the gel when the concentration is less than 2 %. Between concentrations 0·5 and 2 % the relation between coefficient of diffusion and concentration of the gel is roughly expressed by the equation $dk/dc = -0{\cdot}04k$. For concentrations of agar from 2 % upwards the curve approximates more closely to that for gelatin, but it appears to fall less steeply, that is, b is smaller, but the range of 2 to 4 % is too short to admit of accurate determinations of this constant in the case of agar-agar.

3. Despite the greater solidity of agar gels, the calculated coefficient of diffusion generally exceeds that of a gelatin gel of equal strength by about 0·03.

4. The extrapolated values for pure water, using the agar figures, are as follows:

$0°$, $k = 0{\cdot}8 \times 10^{-5}$; $20{\cdot}1°$, $k = 1{\cdot}41 \times 10^{-5}$; $40{\cdot}2°$, $k = 2{\cdot}22 \times 10^{-5}$.

It may be noted that these extrapolated figures are all greater than the constant a in the gelatin results, this constant being the value for the coefficient of diffusion in gelatin of concentration zero. The reason for this divergence is found in the steeper slope of the curve in the case of agar when c is less than 2. The constant a in the equation for gelatin gels is only applicable when the concentration is greater than 2 %. The extrapolated value for k at 20° calculated from Öholm's results is $1{\cdot}31 \times 10^{-5}$.

The ideal method of treating these results would be to compare the values here given for the coefficient of diffusion with those to be derived theoretically from a consideration of the various theories of gel structure. The present extent of our knowledge is however scarcely adequate for this and a first essential is to determine whether the indicator method gives accurate and reliable results. We shall therefore first consider the various possible sources of error in the method, and the magnitude of the errors.

Errors of Measurement.

Mathematical investigation shows that the maximum error due to errors in measurement is 1 % of k, and it is improbable that the error in k due to this cause would exceed 0·3 %, a figure which is trifling in comparison with the gap of 7·5 % between the interpolated values obtained by Öholm and by us for the coefficient of diffusion of sodium chloride in water at 20°.

Errors arising from the conditions of experiment.

In our experiments it is assumed that convection and diffusion keep the concentration of the sodium chloride at the surface of the gelatin constantly at normal concentration. It is also assumed that no evaporation is taking place. As with careful work the values of the coefficient of diffusion calculated from measurements made at different times are the same within the limits of experimental error, it would appear that no serious error arises from these assumptions. Error may also arise from imperfect temperature control. The maximum variation in our experiments was about 0·2°, but generally considerably less than this. An error of 0·2° brings about an error of about 0·5 % in the determination of the coefficient of diffusion.

Theoretical errors.

In deducing the formula from which the coefficient of diffusion is calculated, a number of simplifying assumptions were made to which reference should here be made. The most important of these is that silver nitrate diffuses at the same rate as sodium chloride. It was shown in the second paper in this series [Adair, 1920] that no appreciable error is introduced by this assumption; even doubling the rate of diffusion of silver nitrate has little effect on the

calculated value of the coefficient of diffusion of sodium chloride when the indicator is present in relatively low concentration. The errors arising from simplification of the formula are negligible when N sodium chloride is used and the concentration of the indicator in the gel is only 0·01 N or 0·005 N.

Adsorption of silver nitrate.

Reactions between the silver nitrate and the gel are sources of complication in the phenomena we are investigating, rather than sources of error, but it will be convenient to consider the possibility and consequences of such reactions in this place. Two possible cases will be considered.

1. The silver nitrate forms a reversible compound with the gelatin or agar-agar.

Mathematical investigation [see Adair, 1920, formula IV] shows that if this suggestion is correct, the calculated coefficients are 2·3 % too high.

2. A portion of the silver nitrate forms an irreversible compound with the gelatin.

In this case the calculated coefficients will similarly be too high. The following numbers illustrate the magnitude of the effect produced on the calculated values of the coefficient:

Per cent. of 0·005 N $AgNO_3$ adsorbed ...	2	4	6	8	12	20
Per cent. increase in calculated value of k...	0·49	0·94	1·64	1·89	2·94	4·0

The presented material does not enable us to come to a definite decision on the numerical error in the coefficient of diffusion resulting from adsorption, but some indirect evidence is available.

A glance at the values for the constant b, the rate of decrease in k per unit increase in gelatin concentration, shows that b may be slightly different for gels containing 0·005 N and 0·01 N silver nitrate, though the difference at most is slight, and the recorded difference might be the result of small errors in temperature control or differences in the samples of gelatin used. With such small differences it would appear most improbable that there is any very appreciable removal of silver nitrate from the action of the diffusing sodium chloride.

Conclusion.

A survey of the various sources of error in the determination of the coefficient of diffusion of sodium chloride in gels by the indicator method has yielded the following results:

(1) Errors in measurement: 0·3 % of the value of k.

(2) Errors in temperature control: 0·5 % of the value of k.

(3) Errors due to adsorption: of the order of 1·2 % of the value of k.

These fail to account for the difference between Öholm's extrapolated value for normal sodium chloride at 20°, namely $k = 1{\cdot}31 \times 10^{-5}$, and that given by the indicator method, namely $1{\cdot}41 \times 10^{-5}$, a difference of 7·5 %.

Öholm's value for the coefficient of diffusion of 0·05 *N* NaCl is about the same as the value given for a normal solution by the indicator method. Here we may have the explanation of the apparent discrepancy.

Diffusion coefficients stated for a definite concentration are not strictly accurate. By the ordinary method of determining the coefficients chemical analyses are made of solutions of varying concentration, and for a set of experiments such as that of Öholm, all that can be said with justification is that the coefficients refer to some concentration between the initial value and zero. As the free ions diffuse about twice as fast as the undissociated molecules, the coefficient calculated by the indicator method which measures the rate of penetration of the ions, is bound to be greater than the coefficient calculated on the basis of chemical analyses in which the total quantity of salt, dissociated and undissociated, is considered.

The differences between the values found for the coefficient of diffusion of sodium chloride by different methods could be utilised in an attempt to obtain a more complete description of the diffusion of that salt, but all that can be said at present is that the investigator has a choice of two values of the diffusion coefficient; one if he is rather concerned with the total quantity of salt, and the other if he is concerned with phenomena depending on diffusion gradients near the limit of salt penetration.

Perhaps the most significant fact from the physiological point of view, brought out by work on the diffusion of sodium chloride in gelatin and agar-agar gels, is the smallness of the resistance which even a stiff jelly offers to the diffusing salt. The viscosity of a jelly is of course extremely great, yet the rate of diffusion is reduced by less than 2 % for each gram of gelatin added to 100 cc. when the concentration exceeds 2 %. It has been shown by Einstein [1905, 1906], Sutherland [1905] and von Smolukowski [1906] that k is inversely proportional to η the viscosity, and so we must seek for some explanation of this discrepancy. Although a formula showing the effect of concentration of the gel cannot be deduced at present from the various theories of gel structure [see Lloyd, 1920] a tentative suggestion should perhaps be made with regard to the mechanism which retards diffusion, because this question will require consideration in certain types of diffusion experiments.

The first additions of gelatin to distilled water will have a double effect. There will be (1) an increase in the concentration of ionised gelatin with increase in the gelatin concentration until a maximum value is reached, and (2) an increase in the solid phase. Subsequent additions of gelatin will merely increase the latter. Now it has been shown by Pauli and others that solutions with the greatest proportion of ionised protein have the greatest viscosity, therefore it is unlikely that the (k, c) curve will have the same slope throughout.

The Arrhenius [1892] equation connecting viscosity with coefficient of diffusion, namely $K = K_0(1 - \frac{a}{2}x)^2$, certainly does not agree with the results we have obtained. Öholm also found that the constant "a" of that formula

diminished from 3·6 to 2·3 as the concentration increased. Over the range 2 to 16 % concentration of gel the formula

$$K_c = K_{\text{water}}[0{\cdot}965\,(1 - 0{\cdot}0186\,c)]$$

gives a much better representation of the facts.

It is interesting to compare this result with the value calculated from Einstein's theory [1906] of the viscosity of suspensions of small rigid spheres,

$$\eta' = \eta\,(1 + 2{\cdot}5f).$$

If 1·4 is the specific gravity of the spheres, then a 10 % suspension would reduce k by 15 %. The observed reduction is 18·6 %, and considering that the particles of gelatin are probably arranged in some kind of structure, it is remarkable that the two numbers agree as well as they do, especially as the Einstein formula does not agree exactly with results obtained with the capillary viscometer. Bradford's theory of gel structure [1918] is in agreement with the calculation given above, but the problem is so complicated that the agreement may be no more than a coincidence.

SUMMARY.

The coefficient of diffusion of sodium chloride from normal solution into gels of gelatin and agar-agar of a number of different concentrations has been measured by the indicator method previously described.

The values obtained by extrapolation from these results for diffusion in water are higher than those obtained for the same temperatures by extrapolation from the results of Öholm. The probable reason for this apparent discrepancy is stated.

With increasing concentration of gel, the coefficient of diffusion decreases at first rapidly according to an approximate exponential relation, but with concentrations above 2 % the curve connecting coefficient of diffusion and concentration is approximately a straight line, the rate of decrease of the diffusion coefficient with increasing concentration of the gel being less than with concentrations less than 2 %. It is shown how this result can be harmonised with the relation between diffusion and viscosity found by Einstein, Sutherland and von Smolukowski.

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LXXVI. THE PENETRATION OF ELECTROLYTES INTO GELS.

IV. THE DIFFUSION OF SULPHATES IN GELS.

By WALTER STILES.

From the Department of Botany, University College, Reading.

(*Received August 18th, 1921.*)

Introduction.

It is a curious fact relating to the nutrition of plants that plant tissue appears to absorb chlorides and nitrates to a considerably greater extent than sulphates. This phenomenon appears to be very general. Thus Wodehouse [1917] found chloride abundant in the marine alga *Valonia*, whereas sulphate was absent from it, although abundant in the sea water in which the alga was growing. Stiles and Kidd [1919] found that sulphates were absorbed to a much less extent than chlorides and nitrates by potato tuber and carrot root. The investigations of Pantanelli [1915, 1918] on a large number of plants point to the same conclusion with regard to root absorption. This state of affairs is the more surprising as sulphur is an essential element for the plant, whereas many plants are able to thrive if unprovided with chlorine in any form.

It would appear to be significant in regard to the apparent difficulty of absorption of sulphates by plants as compared with the absorption of chlorides, that a similar difference has been observed in the matter of the penetration of these salts through precipitation membranes [Traube, 1867; Tammann, 1892; Walden, 1892] and membranes of collodion [Brown, 1915].

It thus would seem possible that the small quantity of sulphate that enters living plant tissue as compared with the quantity of chloride and nitrate, might be due to the different degrees of difficulty of sulphates on the one hand, and chlorides and nitrates on the other, in passing through colloidal membranes, of the protoplast [*see* Stiles, 1921]. It becomes of interest, therefore, to determine whether the diffusion of sulphates in gels presents any anomalous features similar to those presented in their diffusion through membranes, which might help to explain the unexpected phenomenon of their comparatively slight absorption by plant tissue.

The only recorded observations on the diffusion of sulphates in gels appear to be those of von Fürth and Bubanović [1918] who used agar-agar containing barium nitrate as an indicator. They did not calculate coefficients of diffusion

from their results, nor do they provide the data to enable this to be done absolutely, but instead they calculate a quantity called by them the "invasion factor," which should be approximately proportional to the square root of the coefficient of diffusion.

From the data of earlier workers von Fürth and Bubanović have calculated the coefficients of diffusion in water at 18° of the substances with which they worked. In Table I are collected the numbers they quote for sulphates together with the invasion factors they found for the same substances and the coefficients of diffusion obtained by taking the squares of the invasion factors.

Table I. Coefficients of diffusion at 18° of some sulphates in water and in 2 % agar-agar containing barium nitrate. (Calculated from the data of von Fürth and Bubanović, the coefficient of diffusion of sodium chloride being taken as unity.)

Sulphate	Invasion factor in gel	Coefficient of diffusion in gel	Coefficient of diffusion in water
Hydrogen sulphate	0·98	0·96	1·33
Potassium sulphate	1·06	1·12	0·74
Zinc sulphate	0·74	0·55	0·48
Magnesium sulphate	0·67	0·45	0·50
Ammonium sulphate	0·67	0·45	0·76

These results exhibit no sort of relation between the coefficients of diffusion in the gel and in water. It has, however, already been pointed out in the first paper in this series [Stiles, 1920], that von Fürth and Bubanović neglect the influence of the concentration of the indicator in their experiments, although this influences very considerably the invasion factor, and consequently the coefficient of diffusion calculated from it. A fresh series of determinations of the coefficients of diffusion of a number of sulphates in gels of gelatin and agar-agar has therefore been made; the values obtained are recorded in a subsequent section of this paper.

Method.

The method employed was that outlined in the third paper in this series [Stiles and Adair, 1921]. Gels of 0·5 % agar-agar and 10 % gelatin were employed, and barium chloride in 0·01 *N* concentration by weight was used as the indicator in the gel. The solutions of sulphates employed were in normal concentration. The experiments were carried out in thermostats maintained at 0° and about 20·5°.

In calculating the coefficients of diffusion from the experimental data, the more exact of the two formulae obtained by Adair [1920] was employed. For this purpose the coefficient of diffusion of barium chloride was assumed to be $0{\cdot}76 \times 10^{-5}$ c.g.s. units under the experimental conditions. Although exact data of this constant do not exist, such values for the coefficient of

diffusion of barium chloride as have been obtained indicate the value used as approximately correct. Even a 50 % error in the value of the constant introduces only a very slight error in the calculated coefficient of diffusion, and it is safe to conclude that any error arising from an assumption of an incorrect value for the coefficient of diffusion of barium chloride is negligible.

Experimental Results.

The sulphates employed were those of ammonium, potassium, sodium, magnesium and copper. The experimental data are summarised in Table II.

Table II. Penetration of various sulphates from a N solution into gels of agar-agar and gelatin containing barium chloride as indicator. (Concentration of barium chloride in the gel, 0·01 N; time in hours; penetration in centimetres of precipitate; diffusion coefficient k in c.g.s. units $\times 10^5$.)

Diffusing salt	Gel	Temperature °C.	Time	Penetration	k
$(NH_4)_2SO_4$	0·5 % agar	0	22·36	2·220	0·474
,,	,,	20·3	23·45	3·202	0·920
,,	,,	,,	47·68	4·599	0·932
,,	10 % gelatin	20·5	22·66	2·748	0·706
,,	,,	,,	46·87	3·955	0·706
K_2SO_4	0·5 % agar	0	22·28	2·218	0·475
,,	,,	20·3	23·44	3·200	0·918
,,	10 % gelatin	20·5	22·67	2·745	0·704
,,	,,	,,	46·90	3·924	0·695
Na_2SO_4	0·5 % agar	0	22·45	1·920	0·355
,,	,,	20·3	23·69	2·937	0·770
,,	,,	,,	47·60	4·213	0·787
,,	10 % gelatin	20·5	22·70	2·515	0·593
,,	,,	,,	46·90	3·615	0·593
$MgSO_4$	0·5 % agar	0	22·12	1·657	0·269
,,	,,	20·0	20·39	2·192	0·502
,,	,,	,,	45·30	3·316	0·517
,,	10 % gelatin	20·5	22·60	2·002	0·384
,,	,,	,,	46·80	2·872	0·382
$CuSO_4$	0·5 % agar	0	22·20	1·793	0·314
,,	,,	20·0	20·25	2·276	0·545
,,	,,	,,	45·23	3·439	0·557
,,	10 % gelatin	20·5	22·74	1·796	0·311
,,	,,	,,	46·95	2·592	0·314

A first glance at these results as they stand shows that the coefficient of diffusion is considerably raised with increase of temperature from 0° to 20°; that at the same temperature diffusion is much more rapid in 0·5 % agar than in 10 % gelatin, and that the coefficients of diffusion of different substances in the gels are roughly parallel to the coefficients of diffusion in water. Each one of these questions will now be considered more in detail.

1. *Influence of temperature.*

If we assume, as is usually done, that the coefficient of diffusion increases with rise of temperature according to a linear relation between diffusivity and temperature, we have the following equations for the coefficient of diffusion of the various salts examined at any temperature θ lying between 0° and 20°.

Salt	Coefficient of diffusion at θ° C.
Ammonium sulphate	$0{\cdot}474\,(1+0{\cdot}0463\,\theta)$
Potassium sulphate	$0{\cdot}475\,(1+0{\cdot}0463\,\theta)$
Sodium sulphate	$0{\cdot}355\,(1+0{\cdot}0592\,\theta)$
Magnesium sulphate	$0{\cdot}269\,(1+0{\cdot}0446\,\theta)$
Copper sulphate	$0{\cdot}314\,(1+0{\cdot}0382\,\theta)$

The increase in the rate of diffusion for rise of temperature of 1° relative to the coefficient of diffusion at 0° thus varies somewhat with different salts, being least for copper sulphate and greatest for sodium sulphate. The numbers are however all of the same order. The value of this quantity in the case of the diffusion of sodium chloride in 0·5 % agar at temperatures between 0° and 20° is however much the same, namely 0·0385, as that for copper sulphate. It is clear that the influence of temperature on the diffusion of sulphates in agar gels presents no unusual features.

2. *Influence of gel concentration.*

In the previous paper of this series the influence of the concentration of the gel on the diffusion of sodium chloride was investigated over a wide range of gel concentrations. In order to determine whether the effect is similar in the case of sulphates it was considered sufficient to carry out experiments with a thin gel of low concentration (0·5 % agar) and a stiff gel of moderately high concentration (10 % gelatin). The values previously obtained with sodium chloride indicate that with that salt the coefficient of diffusion in 0·5 per cent. agar is about 1·3 times that in 10 % gelatin at the same temperature. The ratios of the coefficients of diffusion of the sulphates examined in 0·5 % agar and 10 % gelatin at 20·5° are as follows:

Ammonium sulphate	...	1·31	Magnesium sulphate	...	1·34
Potassium sulphate	...	1·31	Copper sulphate	...	1·78
Sodium sulphate	...	1·32			

With the exception of copper sulphate, the relation between the coefficients of diffusion in 0·5 % agar and in 10 % gelatin is much the same in all the sulphates examined, and in sodium chloride. Copper sulphate forms a very conspicuous exception to the rule in that the coefficient of diffusion is relatively much greater in 0·5 % agar than would be expected from the rate of diffusion in 10 % gelatin.

3. *Comparison of the coefficients of diffusion in gels and in water.*

The observations that have been made in the past on the diffusion of sulphates in pure water have been made at a number of differing temperatures

and with very varied concentrations of diffusing salts. A comparison between the actually observed rates of diffusion in gels and water cannot therefore be made. However from the results obtained for the coefficient of diffusion of sulphates in 0·5 % agar at 0° and 20°, it is at once possible to obtain by interpolation the values for the coefficient of diffusion in this gel at the temperatures used in earlier investigations on diffusion in pure water. In the following Table are shown, for the sulphates examined in this investigation, the values of the coefficients of diffusion in pure water and in 0·5 % agar-agar at the same temperature. The concentrations of the diffusing salts are not always the same, but the coefficients of diffusion in pure water tabulated are those for the concentration of salt nearest to normal, that used in the experiments with gels. Observations made by Öholm [1905] on other salts suggest that generally decrease in concentration results in an increase in the coefficient of diffusion, but the influence of concentration is not very great over small ranges of concentration.

Table III. Comparison of the coefficients of diffusion of various sulphates in water and in 0·5 % agar-agar. (Coefficient of diffusion, k, in c.g.s. units $\times 10^5$; concentration of salt in diffusion in gels N in all cases; concentration of salt in diffusion in water given in normalities in fifth column.)

Diffusing salt	Temperature ° C.	k in gel	k in water	Concentration in normalities of salt in diffusion in water	Observer of k in water
$(NH_4)_2SO_4$	18	0·869	0·880	2·0	Lang—Hedin—von Fürth and Bubanović
K_2SO_4	19·6	0·903	0·995 0·914	0·56 1·9	Thovert
Na_2SO_4	10·4	0·574	0·567	2·58	Graham-Stefan
$MgSO_4$	5·5	0·335	0·37	1·08	Scheffer
	10	0·389	0·394	0·80	
$CuSO_4$	17	0·518	0·336	1·00	Thovert

The numbers presented in this Table show conclusively that, with the exception of copper sulphate, the values for the coefficient of diffusion of the sulphates in 0·5 % agar are very close to those for diffusion of the same salts in pure water. The evidence is that the coefficient in agar is slightly less than in pure water, as would be expected from the increased viscosity in the gel.

Conclusion.

From the results of this study of the diffusion of a number of sulphates in gelatin and agar-agar gels, it appears evident that, with the single exception of copper sulphate, the diffusion of sulphates in gels presents the same features as that of sodium chloride. Diffusion proceeds almost as rapidly in 0·5 % agar-agar as in pure water, while in 10 % gelatin the diffusion coefficient is reduced about 25 %. The influence of temperature is similar to that on the coefficient of diffusion of sodium chloride. Copper sulphate, on the other hand, shows

an abnormally large coefficient of diffusion in 0·5 % agar-agar, although the coefficient of diffusion of this salt in 10 % gelatin bears more nearly, though not exactly, the same relation to the coefficient of diffusion in water as the other salts examined. This is a very curious phenomenon for which at present no explanation can be offered, but which certainly deserves further investigation.

A reference to Table I of this paper shows that the results of von Fürth and Bubanović indicate that the coefficients of diffusion of potassium sulphate and zinc sulphate are greater, and that of ammonium sulphate considerably less, in a 2 % agar-agar gel than in pure water. The more exact determinations recorded in this paper show clearly that such a divergence between diffusion of these sulphates in water and in gels does not exist.

The comparatively small intake of sulphates by plant tissue is therefore not to be explained on account of any abnormal behaviour of these salts in diffusing through colloidal systems. Further evidence in this direction is forthcoming from the observations of Stiles and Kidd [1919] in which it was shown that the plant tissue examined absorbs sulphates *initially* faster than either chlorides or nitrates, but that the rate of absorption of sulphate falls off very much more rapidly than that of chloride or nitrate. It would thus appear that it is a question of equilibrium in a chemical combination, or in adsorption, that limits the intake of sulphates, rather than any retardation owing to a particularly slow rate of diffusion, or to a change brought about by the sulphate on the colloidal system as such which renders it much more resistant to the passage of the ions.

Summary.

The coefficients of diffusion of a number of sulphates in 0·5 % agar-agar and 10 % gelatin have been determined by the indicator method. In the cases of the sulphates of ammonium, potassium, sodium and magnesium the coefficient of diffusion in 0·5 % agar-agar is very close to, though slightly less than, that in pure water at the same temperature and for the same concentration of diffusing salt, while the coefficient of diffusion in 10 % gelatin is about 25 % less than in 0·5 % agar-agar. The diffusion coefficient of copper sulphate in 0·5 % agar-agar is considerably greater than in water, but in 10 % gelatin it is smaller than in water but yet somewhat higher than the values obtained for other sulphates would indicate. Increase of temperature from 0° to 20° increases the diffusion coefficient about 1·76 to 2·2 times according to the salt. These effects are of the same order as in the case of sodium chloride.

Except in the case of copper sulphate the diffusion of sulphates in the gels examined proceeds normally and the results of von Fürth and Bubanović indicating the contrary are not confirmed.

The comparatively small uptake of sulphates by plant tissue is not to be explained on the basis of retarded diffusion through a gel.

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LXXVII. A METHOD FOR THE SEPARATION OF AMINO-ACIDS FROM THE PRODUCTS OF HYDROLYSIS OF PROTEINS AND OTHER SOURCES.

PRELIMINARY COMMUNICATION.

By HAROLD W. BUSTON and SAMUEL BARNETT SCHRYVER.

From the Biochemical Department, Imperial College of Science and Technology.

(*Received August 30th, 1921.*)

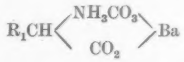
In the course of investigations on the nitrogenous metabolism in green leaves, experiments were undertaken with the object of isolating the various nitrogenous substances contained in cabbages. It has already been shown by Chibnall and Schryver [1921] that nearly all the simpler nitrogenous products can be removed from the leaves when they are treated with water saturated with ether, and the liquid is then expressed in a tincture press. An opalescent solution is thereby obtained, which on warming to 40° deposits a flocculent precipitate, consisting for the most part of proteins. After filtration and warming further to 100° another fraction of proteins is precipitated. On adding an equal bulk of 95 % alcohol to the filtrate from this precipitate, a voluminous pale buff-coloured precipitate is produced, containing in addition to inorganic salts and other substances which account for only a relatively small percentage of the total nitrogen, an organic phosphate derivative which has not yet been further investigated. The filtrate from this phosphate fraction was concentrated under diminished pressure, acidified with sulphuric acid, and the requisite amount of phosphotungstic acid was added. 32·8 % of the nitrogen in the extract after removal of the protein and phosphate fractions was thereby precipitated; this extract contained also 11·4 % of its nitrogen in the form of ammonium salts. The filtrate and the washings from the phosphotungstate precipitate were treated with barium hydroxide (Congo red) to remove the greater part of the sulphuric acid and excess of phosphotungstic acid, and then evaporated *in vacuo*. The liquid thus obtained contained 53·55 % of its nitrogen in the amino form (Van Slyke). Experiments were then made to remove the dicarboxylic acids by barium hydroxide and alcohol. The first addition of the alkali produced a precipitate of phosphotungstate containing no nitrogen; this was filtered off, and a further addition of barium hydroxide in saturated solution was made, and then $1\frac{1}{4}$ the bulk of 95 % alcohol was

added. A precipitate of the dicarboxylates was thereby produced. It is not advisable to add a larger bulk of alcohol, otherwise the precipitate tends to become slimy. 13·9 % of the nitrogen in the extract (after removal of proteins and phosphates) is thereby precipitated.

After the separation of the barium dicarboxylates, carbon dioxide was passed into the liquid, *without previous removal of the alcohol*. A bulky precipitate was thereby produced, which contained appreciable quantities of nitrogen (1·66 % of the dry weight). This was filtered off, and the filtrate was saturated with barium hydroxide, and treated with carbon dioxide. Another precipitate was obtained, which was also relatively rich in nitrogen. These experiments were repeated with a larger bulk of the liquid. An extract freed from the dicarboxylic acids and alcohol was diluted with an equal volume of 95 % alcohol, and saturated with solid barium hydroxide; somewhat more was required than would saturate the amount of water present. Carbon dioxide was then passed through the solution. The precipitate was filtered off, the filtrate was re-saturated with barium hydroxide, and again treated with carbon dioxide. This treatment was repeated altogether seven times, and the precipitates contained the following percentages of nitrogen: (i) 1·70, (ii) 1·67, (iii) 0·92, (iv) 0·81, (v) 0·55, (vi) 0·29, (vii) 0·09. Altogether, 68·83 % of the nitrogen contained in the liquid from which the bases and dicarboxylic acids had been separated by the methods given above were thus removed. Furthermore, the residual liquid contained in the amino form only 2·6 % of the total nitrogen present in the liquid after removal of the dicarboxylates.

Determination of the optimal conditions of precipitation.

Many years ago Siegfried prepared the barium salts of carboxylates of amino-acids, possessing the general formula



by treating these acids with carbon dioxide and barium hydroxide. These barium salts readily decompose on warming, yielding barium carbonate and the free amino-acids. There is little doubt that the amino-acids have been precipitated in the form of the barium salts of their carboxylates by the method described above. Experiments were undertaken with the object of determining the optimal conditions of precipitation, a few of which are given in detail below.

After addition to the base-free extract of cabbage of the amount of barium hydroxide necessary to precipitate the barium salts of the dicarboxylic acids after addition of alcohol, the filtered liquid was concentrated *in vacuo*. To the liquid thus obtained varying quantities of alcohol and barium hydroxide were added before treatment with carbon dioxide. The gas was then passed

in and the amount of precipitate produced, and its percentage of nitrogen were determined. The results are given in the following table:

(*a*)	1000 cc. alcohol; 1000 cc. extract: 15 g. $Ba(OH)_2$	Weight of precipitate	12·5 g.; N 1·61 %.
(*b*)	500 cc. alcohol; 500 cc. extract: Baryta to saturation	,, ,,	24·5 g.; N 1·70 %.
(*c*)	200 cc. extract; 400 cc. water; and 600 cc. alcohol		
	(i) 35 g. $Ba(OH)_2$	,, ,,	31·5 g.; N 1·60 %.
	(ii) 30 g. $Ba(OH)_2$	,, ,,	N 0·77 %.
(*d*)	200 cc. extract; 400 cc. alcohol: 10 g. $Ba(OH)_2$	,, ,,	9·5 g.; N 1·60 %.
(*e*)	200 cc. extract; 0 cc. alcohol: 30 g. $Ba(OH)_2$	,, ,,	21·5 g.; N 0·35 %.

As a result of the above experiments it will be seen that very little nitrogenous matter is precipitated by successive treatments with barium hydroxide and carbon dioxide in the absence of alcohol; on the other hand no advantage is obtained by diluting the solution from which the amino-acids are to be precipitated with more than an equal bulk of 95 % alcohol. By using therefore an equal bulk of alcohol and adding the baryta to saturation before treatment of the liquid with carbon dioxide, the best results are to be obtained. Under these conditions the results recorded in the preceding section were obtained from the cabbage extract.

The main disadvantage of the method lies in the fact that the alternate treatment of the liquid with barium hydroxide and carbon dioxide must be repeated several times before a nitrogen-free precipitate is obtained. To obviate the manipulations necessary for this process, an attempt was made to convert the amino-acids into their carboxylates by treatment with an excess of ethyl chloroformate in the presence of sufficient barium hydroxide to combine with the hydrochloric acid set free in the reaction, and the subsequent addition of a large excess of baryta. Several experiments of this character were carried out, but in no case did the precipitate obtained contain more than small amounts of nitrogen, and, compared with the first methods tried, this alternative method showed no advantages. Furthermore, it was found that after a little practice, when the amounts of reagents necessary were known, the successive operations of the earlier methods could be carried out without undue expenditure of time.

Decomposition of the nitrogenous barium precipitate.

150 g. of the precipitate containing 1·66 % of N (or 2·49 g. in all) were decomposed by boiling for 5–10 minutes with water; a Kjeldahl estimation on the filtrate showed that over 98 % of the nitrogen present in the precipitate was dissolved by this method. The brown-coloured filtrate was found to contain considerable amounts of barium in solution; this was accordingly removed *exactly* with sulphuric acid. The filtrate was decolorised by means of a little animal charcoal, and evaporated *in vacuo* to syrup, of amber colour. On

shaking this syrup with absolute alcohol it became changed to a cream-coloured, granular solid, while the alcohol became light orange-brown in colour. The solid was removed, washed two or three times with absolute alcohol, and finally with ether, and dried by leaving for two or three hours in a vacuum desiccator. By this method, it dried to a light buff powder, easily soluble in water, and containing 5·25 % of nitrogen. (Unless the washing with alcohol was thorough, and the drying rapid, the substance tended to change back to an amber-coloured, sticky mass.) Evaporation of the alcoholic extract gave a brown, non-crystalline mass. Judging from the percentage of nitrogen in the solid (*i.e.* 5·25 %), it appears that it contains about 40–50 % of amino-acid; the remainder consists probably of sugars and possibly oxy-acids. Further experiments to decide this point are to be made.

From the results of the above experiments it was found that a relatively large fraction of the simpler substances from the cabbage leaves could be isolated in a crystalline and but slightly pigmented form, and almost completely free from the resinous matters which hitherto have made the investigation of the crystalline substances contained in leaves a problem of great difficulty. The investigation of these substances of which a large proportion are nitrogenous is reserved for the future. It is worthy of note that leaves of plants contain relatively large amounts of nitrogenous substances which are neither bases nor amino-acids. The discovery of a method which removes nearly all these acids should considerably simplify the investigation of these unknown constituents.

In the meantime, it was thought advisable to ascertain whether the baryta-carbon dioxide method could be applied to the isolation in crystalline form of the amino-acids obtained by the hydrolysis of the proteins.

The discovery by Dakin of the butyl alcohol method forms a great advance in the technique of the isolation of the amino-acids, but this is not always convenient, as Dakin's recently published paper on the hydrolysis products of gelatin shows [Dakin, 1921]. It seemed that the method described in this communication might serve as an alternative one in such cases; it has the further advantage that it can be carried out much more rapidly than the butyl alcohol extraction method. The experiments described below on the isolation of the hydrolysis products of caseinogen and gelatin indicate that the method described in this paper is one of considerable promise.

Experiments with hydrolysed caseinogen.

The caseinogen was hydrolysed with sulphuric acid in the usual way; sulphuric acid was removed by means of baryta, and the liquid evaporated to small bulk to remove tyrosine. After removal of tyrosine, the amount of nitrogen present was 8·870 g. The concentrated liquid was diluted and precipitated with phosphotungstic acid in the ordinary way; 2·50 g. of nitrogen were thus removed, leaving 6·37 g. in solution. Dicarboxylic acids were now removed by treating the solution (concentrated to 500 cc., after removal of

excess of phosphotungstic and sulphuric acids) with 35 g. baryta, adding 1000 cc. alcohol, and allowing to stand for 20 hours. The product which crystallised out was removed, washed with 70 % alcohol and dried; its weight was 27 g., and it contained 1·6 g. of nitrogen.

The filtrate was concentrated to 250 cc. and contained 4·65 g. of nitrogen, of which 68·27 % (or 3·168 g.) was in the form of amino nitrogen. Of this solution, 125 cc., containing 2·325 g. of nitrogen and 1·584 g. amino nitrogen were used; baryta was added to saturation, then an equal bulk of 95 % alcohol, and CO_2 passed till precipitation was complete. The precipitate produced was of a gelatinous nature and gave some difficulty in washing and filtering; the gelatinous nature is less obvious in the fractions containing a lower percentage of nitrogen. Four such precipitations were made, and the amount of nitrogen brought down in each case was determined; the residual liquid was evaporated to a small bulk *in vacuo*, and the nitrogen remaining determined. The results are summarised in the Table below. A van Slyke determination on the residual solution gave 0·1175 g. amino nitrogen present, or 5·06 % of the total original nitrogen.

Each of the above four fractions of the barium precipitate was decomposed separately by boiling up twice with three times its weight of water for 5 minutes; after filtering and removing barium from the extract by means of the exact amount of sulphuric acid, the liquid was in each case examined for amino nitrogen. The results obtained are shown in the following table:

Caseinogen: solution after removal of dicarboxylic acids.

	Total N (Kjeldahl) g.	%	Amino N (van Slyke) g.	%	Non-amino N (by difference) g.
Original solution	2·325	100·00	1·584	68·27	0·741
Fraction I	0·906	39·00	0·6786	29·19	0·2274
„ II	0·545	23·47	0·3995	17·18	0·1455
„ III	0·298	12·79	0·2095	9·01	0·0885
„ IV	0·275	11·85	0·1702	7·31	0·1048
Residue	0·280	12·04	0·1175	5·06	0·1625
Totals (compare with top line, "Original solution")	2·304	99·15	1·5753	67·75	0·7267

(N.B. In the above table, all percentages given are calculated as percentage of the total nitrogen present at the commencement of the series of experiments, 2·325 g.)

The solutions from decomposition of the barium precipitates, after the above determinations had been carried out, were evaporated to small bulk *in vacuo* till quite syrupy. The syrups were then shaken with a large bulk of absolute alcohol, when they were converted to pinkish-white granular masses; complete solidification is difficult to obtain unless the syrups be practically anhydrous. (A small portion of the solid was removed from alcohol, dried at 110°, and the nitrogen estimated—N = 10·84 %. The bulk of the

product was not dried down.) The nitrogen present in the solid product was entirely amino nitrogen; a portion of the alcohol was evaporated *in vacuo* and gave a semi-crystalline yellow mass.

Experiments with hydrolysed gelatin.

Precisely similar experiments to those carried out on caseinogen were also performed in the case of gelatin; the main object was to test the completeness of removal of amino nitrogen from solution, as the method of H. D. Dakin, using continuous extraction with butyl alcohol, breaks down when glycine is present in large quantity. Exact details of the method adopted need not be given here, as they were precisely the same as those described above. The main results were as follows:

Weight of nitrogen in hydrolysed liquid = 21·25 g.
" " after phosphotungstate treatment = 13·65 g.
" " " removal of Ba dicarboxylates = 12·424 g.
" " as amino nitrogen = 9·792 g.
= 78·81 % of total N present after removal of Ba dicarboxylates

Only one-fourth of the solution at this stage (*i.e.* 100 cc. containing 3·106 g. of nitrogen) was worked up. The percentage of nitrogen contained in the first fractions of the barium precipitate was much higher than in the case of cabbage and caseinogen precipitates; the end point of the precipitation was, however, of the same order—87·73 % of the total nitrogen being removed (compared with 87·96 % in the caseinogen experiment).

The percentages of nitrogen in the four successive fractions of the barium precipitate were: 5·61 %, 2·30 %, 0·66 %, 0·29 %. Fraction I was decomposed and the extract examined separately; fractions II and III were combined and decomposed, etc.; fraction IV was decomposed and examined separately as before, the residual liquid was evaporated to small bulk and examined for total and amino nitrogen. The results are summarised below.

Gelatin: $\frac{1}{4}$ of solution after removal of dicarboxylates.

	Total N		Amino N		Non-amino N
	g.	%	g.	%	g.
Original solution	3·106	100·00	2·448	78·81	0·658
Fraction I	1·814	58·40	1·702	54·79	0·102
" II, III	0·907	28·13	0·644	19·93	0·263
" IV	0·015	0·48	—	—	—
Residual liquid	0·363	12·27	0·073	2·42	0·290
Totals (cf. top line "Original solution")	3·099	99·28	2·419	77·14	0·655

It will thus be seen that the removal of amino nitrogen by this method is almost complete in the case of gelatin.

The amino-acids themselves were obtained as before by evaporation of the solution from the decomposed barium precipitate *in vacuo* and treatment with absolute alcohol. The first fraction easily set to a white crystalline product, containing 12·96 % N, all in the form of amino nitrogen. The second fraction took much longer to granulate, being, after a week, still in a somewhat pasty form; this was due probably to incomplete drying in the vacuum desiccator. A small portion, dried at 105°, gave N = 11·56 %.

No experiments were made on the nature of the substances extracted by alcohol from the amino-acids; probably they were proline substances, which Dakin has shown to be present in large amount among the hydrolysis products of gelatin.

From the results given above, there can be little doubt that the baryta-carbon dioxide method can be applied to the isolation in crystalline form of the hydrolysis products of the proteins. In the above described experiments the separation of the amino-acids is not quite complete, but it is probable that it could be rendered more so by the addition of baryta and carbon dioxide to the filtrate from the four precipitations after this has been concentrated. It will be noticed that this residual fluid contains a relatively large amount of the nitrogen in the non-amino form.

It is proposed to investigate the method in greater detail, and to determine, amongst other things, whether it can be applied to a partial separation of the amino-acids from one another. It is also possible that it may be used for separating the diamino-acids from other bases. In view of the amount of time the various subsidiary investigations mentioned in this paper will take to complete, it has been thought advisable to publish a short account of some of the results obtained up to the present date.

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LXXVIII. THE PECTIC SUBSTANCES OF PLANTS. PART II. A PRELIMINARY INVESTIGATION OF THE CHEMISTRY OF THE CELL-WALLS OF PLANTS.

By DONALD HERBERT FRANK CLAYSON,
FREDERICK WALTER NORRIS
and SAMUEL BARNETT SCHRYVER[1].

A Report to the Food Investigation Board.

From the Biochemical Department, Imperial College of Science and Technology.

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In a previous communication [Schryver and Haynes, 1916] it was shown that by treating the insoluble portions of plant tissues with warm ammonium oxalate solution, a substance was extracted which could be precipitated in the form of a voluminous gel on addition of alcohol. This was the salt of an acid to which the name "pectinogen" was provisionally assigned. This acid was shown to be soluble in water and to give a soluble calcium salt, but was converted into another product on keeping for a short time with *N* NaOH, which was designated "pectin." This is a product of acidic nature; the free acid is insoluble in water, gives an insoluble calcium salt, and readily forms a jelly when treated with normal NaOH. Von Fellenberg [1918] subsequently showed that pectic substances when treated with caustic alkalis in the cold give rise to methyl alcohol.

The present investigations were undertaken with the object of throwing some light on the relationship between pectinogen and pectin, and of ascertaining the form in which the pectic substances are combined in the plant tissues.

In the first instance it was proposed to repeat von Fellenberg's work with the primary object of determining whether there is any quantitative relationship between the amount of alcohol set free by sodium hydroxide from the insoluble portion of the plant tissues, and the amount of pectic substances contained therein. In the second instance, in view of the fact that a primary substance, "pectinogen," can be isolated from the tissues, which is changed by alkalis into "pectin," experiments were undertaken to ascertain whether this latter substance could be extracted directly by means of cold caustic alkalis.

[1] The above work was commenced in conjunction with Mr C. C. Wood. Owing to his acceptance of a new position he had to relinquish the work, and his place was taken by Mr D. H. F. Clayson. The authors are indebted to Mr Wood for the assistance given in this work.—S. B. S.

As a result of the experiments it was found, in all the tissues investigated, that *N* sodium hydroxide causes the elimination of methyl alcohol and also extracts another substance or mixture of substances in relatively small quantities which have properties quite distinct from the pectic substances, and are more closely allied to products which have been called by Schulze the "hemicelluloses." They can be precipitated from the alkaline solution by means of alcohol as bulky flocculent precipitates; they give a blue coloration with iodine, do not directly reduce Fehling's solution, but are readily hydrolysed by quite dilute mineral acids giving solutions which readily reduce the above-mentioned reagent. On treatment with concentrated hydrochloric acid they give rise to furfural, and the amounts obtained from different sources vary within wide limits, the quantities corresponding to pentose contents ranging between about 40 % and 85 %. It is proposed to designate these products, which are probably reserve carbohydrates of the tissues, as *cytopentans*. Although extracted from the tissues by alkaline solutions, in which they readily dissolve, they are not of distinct acidic nature, and are only in isolated cases precipitable in flocculent form on addition of acids.

As already mentioned, the alkaline extract which contained methyl alcohol and the "cytopentans" did not hold in solution (provided carbonate was not present in the caustic solution in appreciable quantities) any pectic substance. This however could be extracted from the residues obtained after treatment with caustic alkalis by treatment with *warm* ammonium oxalate solution. On adding acid to the ammonium oxalate extract, a gelatinous precipitate was immediately thrown out which corresponded in all its properties with the product previously designated as "pectin." To avoid confusion in the nomenclature it is proposed to call this product, which appears to be a general constituent of the cell walls and is found in variable quantities, "cytopectic acid." Such a special name seems desirable in view of the fact that the name pectin is generally given to the jelly-forming substance which is obtained in quantity from only a limited number of sources, and is utilised commercially in the manufacture of preserves.

The cytopectic acid was prepared in the course of the present work from six different sources. The product obtained in all cases was so similar, alike in the optical rotation, the amount of furfural yielded on distillation with hydrochloric acid, and in the carbon and hydrogen content, that there is strong reason to believe that the acid obtained from all the sources hitherto examined is the same. Only in the case of the orange were the numbers obtained for the carbon percentage distinctly below the general average, and the optical rotation was about 20° lower than the average obtained for the cytopectic acid preparations from other materials. The oranges, however, differed from the other products in that the expressed juices were very rich in soluble pectic substances and set soon after they were obtained to a solid jelly. It is conceivable that the soluble pectins are derived from the pectic substances of the cell wall, and that the latter undergo some kind of change on

yielding the former. The investigation of the soluble pectic substances has, by arrangement with the authors, been left to Professor Barker and his colleagues, whose researches may throw further light on this question. The present work has been confined mainly to the ascertainment of the general distribution of the products described above in the insoluble portions of the plant tissues.

There was not found to be any constant ratio between the methyl alcohol obtained by the treatment of the cell-wall substances with cold sodium hydroxide solutions, and the amount of cytopectic acid contained therein.

After the elimination of methyl alcohol, the cytopentans, and cytopectic acids by the methods described above, a residue was obtained which was almost completely soluble in the ordinary reagents used for the solution of celluloses (such as zinc chloride and hydrochloric acid, or Schweitzer's reagent). Four products have therefore been obtained from all the cell-wall substances examined. The percentages of methyl alcohol, cytopentans and cytopectic acid are given in the following table:

	Methyl alcohol	Cytopentans	Cytopectic acid
Turnips	0·23	2·8	18·3
Onions	0·16	1·2	13·5
Pea-pods	0·21	5·4	10·4
Cabbage	0·27	0·9	18·0
Oranges	0·42	1·8	26·0
Apples	0·35	1·4	6·0

The above numbers are calculated on the dry weights of all the substances.

In this communication it has been possible to deal only with the outlines of the subject of the composition of cell-wall substances, and a number of problems are presented which call for further investigation. A few may be mentioned here. It would be of interest to ascertain whether the methyl alcohol, which is eliminated so readily from the tissues, is a product of the photochemical reduction of carbon dioxide. Materials for experiments on this question are in course of preparation.

The exact form in which the cytopectic acid is contained in the tissues remains to be decided. It appears to be in some form of chemical combination with the cellulose, and is only extracted by *warm* solutions of salts, the anion of which gives insoluble calcium salts, such as sodium carbonate, ammonium oxalate and tartrates, but not by other salts such as sodium chloride. Further investigations are also necessary to ascertain definitely whether the cytopectic acid from all sources is the same chemical individual. The relative distribution of the four constituents of the cell-wall substances remains also to be determined. It will be of especial interest to discover whether the relative proportions vary during the different stages of the life histories of the tissues, such as for example, during the ripening of fruits, a subject of some practical importance in view of the work being carried out in the biological departments of the College on the cold-storage of fruits.

Experimental.

A. *Preparation of the Materials.*

The material used for the elementary stages of the work was obtained from turnips. Owing to the difficulty experienced in dealing with the colloidal products, it became necessary to evolve a technique for their preparation, and a large quantity of the dried turnip residues was prepared for this purpose.

The method of working up these residues from the fresh product and of the pectic substances from them may be described in some detail, as the method finally adopted is a general one, with but few modifications in special cases, which will be indicated.

In the case of the turnips, 15 kilos represented one batch used. The fresh vegetable was cleaned free from any surface mould, etc., cut up into small pieces and passed through a mechanically driven mincer, using as fine a plate as possible. The minced product was thoroughly pressed in a tincture press as quickly as possible and then placed in clean enamelled basins with a large quantity of distilled water containing chloroform, which acted both as a cytolysing agent and antiseptic. It is essential to wash and press many times to ensure that all the liquid extracts are eliminated. After about ten alternate washings and pressings, the liquid came through almost clear and the residues were further subjected to a final pressing and then spread out and dried by a current of air from a fan, for two or three days. When dry, they may be ground if necessary, but most of the products used were in a sufficiently fine condition after mincing. First results seemed to indicate that the yield of cytopentans, for example, was affected by the state of division of the residues, but subsequently the main factor was seen to be that of imbibition. If the residues are allowed to stand with water previous to the chemical action of soda or oxalate, they swell very considerably and become completely impregnated with water. The chemical is thus enabled to attack the whole of the fibre and irregular action is minimised.

In the case of the turnips the residues are a light yellow in colour, resembling sawdust. They may however be ground down to an extremely fine dust. 600 g. of dried residues were obtained from the 15,000 g. of the fresh product.

Before extraction of the pectic bodies, the residues were boiled with alcohol which removes a quantity of pigment. Afterwards they were thoroughly washed with a large quantity of distilled water and again air-dried.

The other fresh products were worked up in similar fashion and in the case of oranges, apples and onions the alcohol extraction is very necessary, as this removes besides pigments, essential oils, etc. This extraction and the very thorough washing with distilled water which should follow are particularly necessary from the point of view of the methyl alcohol determination.

A modification was made in the case of cabbages. The high protein content is somewhat disturbing from the present point of view, and in order to eliminate as much nitrogenous material as possible, Chibnall and Schryver's [1921]

method of extraction with ether water was employed. The minced product was allowed to stand with ether water for about 20 mins. and then pressed. This treatment was repeated and then the washings and pressings carried on as before.

B. *Estimation of the Methyl Alcohol.*

The methyl alcohol content of the dried fibre residues was determined by the method of Schryver and Wood [1920].

The dried residues, prepared as described, were boiled with alcohol, if in the course of the preparations this had not been done, and afterwards thoroughly washed and pressed with many changes of water to ensure complete elimination of alcohol, essential oils and colouring matter.

A quantity of the thoroughly dried residues—from 2 to 3 g.—was weighed out and placed in a clean dry Erlenmeyer flask. 50 cc. of water were then added and allowed to stand for $1\frac{1}{2}$ hrs. The residues imbibed the water and thus were more readily attacked by 50 cc. of 2 *N* NaOH, which were then added and allowed to stand for $1\frac{1}{2}$–2 hrs. The soda extract was then rapidly filtered through filter paper (previously shown to be free from methyl alcohol) into another clean dry flask and 25 cc. of this were then placed in a distilling flask, and the methyl alcohol distilled off under diminished pressure. The caustic soda was first neutralised with phosphoric acid, and a dropping funnel and capillary tube were fitted to the flask. The flask was placed in a water-bath and the side tube connected with a worm which was surrounded by crushed ice, and terminated in the flask, which was surrounded by a bath of ice and salt and thus kept at about – 10° C. Another flask, placed between the former flask and the pump, also surrounded by a freezing mixture, served as a trap for any of the distillate which escaped condensation. Finally connection was made with a pump and manometer. In practice, this apparatus was used in duplicate. The distillation was conducted at as low a temperature and pressure as possible. After some preliminary frothing, boiling proceeded quietly and when the original 25 cc. was almost all distilled over, 20 cc. of distilled water were added, and when this was evaporated a final 20 cc. were added. Finally the tubes were washed through with distilled water and the frozen distillate allowed to melt and then made up to 250 cc. with distilled water. The methyl alcohol was then estimated by the method of Schryver and Wood above mentioned.

The following percentages of methyl alcohol were found in the dried residues:

Turnips	Onions	Pea-pods	Cabbages	Oranges	Apples	
0·22	0·17	0·205	0·278	0·424	0·365	
0·23	0·15	0·218	0·275	0·415	0·344	
0·22	0·16	—	—	—	—	
0·24	—	—	—	—	—	
0·23	0·16	0·21	0·27	0·42	0·35	Averages

These results show no definite ratio between the methyl alcohol and the cytopectic acid.

C. *Preparation and Properties of the Cytopentans.*

It has been noted that sodium carbonate or carbon dioxide was capable of extracting cytopectic acid from the residues. Hence when it was found that caustic soda extracted a substance which differed from cytopectic acid, precautions were adopted to prevent formation of carbonate and consequent extraction of the cytopectic acid.

The procedure may be indicated in detail in the case of the turnip cytopentan. 300 g. of the dried residues were placed in each of two jars fitted with stoppers and rubber washers to exclude air. 3000 cc. of air-free distilled water were added to each and allowed to stand for $1\frac{1}{2}$ hrs. At the end of this period all the water had been imbibed and a moist mass, swollen considerably, remained. To each then were added 3000 cc. of 8 % caustic soda made up from purest fresh sticks and air-free distilled water. This was allowed to stand for a further $1\frac{1}{2}$–2 hrs.; the soda extract was then rapidly filtered off through muslin on a large Buchner funnel and the residues pressed very thoroughly. It was found that no further cytopentan was extracted if the above treatment were repeated, and accordingly the residues were plunged in large quantities of distilled water to wash the remaining soda from the fibres. It is essential to eliminate all caustic soda, as otherwise the subsequent extraction of cytopectic acid with ammonium oxalate is rendered useless, as the colloid will come out contaminated and in a condition impossible to work up to a dry condition.

The soda extract obtained above was filtered again through filter paper pulp and placed with an equal bulk of 95 % alcohol, in Winchester bottles. The yellow extract became cloudy on addition of the alcohol, and a white flocculent precipitate was gradually formed. Filtration was found to be tedious, wasteful and generally inefficient. The method finally adopted was as follows. If the extract and precipitate are thoroughly shaken, air bubbles are released from the precipitate, and on standing overnight this sinks to the bottom of the jar or bottle; the supernatant liquid may then be almost completely syphoned off, using a small muslin bag over one end of the syphon to prevent undue loss of cytopentan. The remaining liquid and precipitate was then centrifuged. The cytopentan at this stage was a slimy white product. It was allowed to stand in 50 % alcohol for a day and then centrifuged; the alternate treatment with 50 % alcohol and centrifugalisation was carried on until the alcoholic washings no longer showed alkaline to litmus. The strength of the alcohol was then increased after each separation until finally the cytopentan presented itself as a white granular substance in absolute alcohol. Washing with ether followed, and finally the product was allowed to dry in a vacuum desiccator.

This method of preparation was adopted in every case and the resulting cytopentans were fine powders varying in colour from white or yellowish to grey.

Before carrying out any estimations, it was considered advisable to reprecipitate the product from solution, the object being to remove any cytopectic acid inadvertently carried down and also if possible to reduce the ash.

To this end the product was dissolved, in the proportion of 1 g. to 50 cc., in 4 % caustic soda, a little water being added if necessary. Solution takes place slowly in the course of a day. Acetic acid was added until the mixture was just neutral, and a few drops of calcium chloride were then added to carry down the very small traces of cytopectic acid as a gel of calcium salt. On centrifuging, a small quantity of foreign matter and a gel came down. The supernatant liquid was decanted and filtered, the cytopentan precipitated by alcohol and worked up through graded strengths of alcohol as before.

The cytopentans give a blue coloration with iodine, similar to that of starch. They do not reduce Fehling's solution directly, but are readily hydrolysed by 1 % hydrochloric acid to give products which reduce Fehling's solution.

They contain large and varying quantities of pentose, as shown by Tollens' phloroglucinol method.

Estimation of Pentose in Cytopentans.

The reprecipitated products were very carefully dried first in a current of air and then in vacuo over phosphorus pentoxide at 100°. About 0·2 g. of the dry cytopentan was weighed out into a small glass capsule and placed in a distilling flask together with 100 cc. 12 % hydrochloric acid, and the phloroglucinide obtained in the usual manner. The black precipitate was filtered through a clean weighed alundum crucible and washed with 150 cc. water; after drying at 105° for 4 hours it was weighed.

It has been shown by Ellett and Mayer that methylpentose may be distinguished from pentose owing to the fact that the methylfurfural phloroglucinide is soluble in hot alcohol, and the furfural compound is not. In accordance with this, the precipitate was extracted twice with hot alcohol and the crucibles finally weighed after two to three hours drying at 100°. It was found that a very small quantity was extracted by the alcohol, varying within wide limits, 0·6–6 % of the cytopentan as shown in the table. Further, the percentages of pentose calculated on the weight of precipitate before extraction varied within the same wide limits; after extraction, however, a much cleaner precipitate remained and the pentose values on this basis were much more concordant.

Before discussing the results obtained it is necessary to point out that these cytopentans are probably mixtures or at least variable products of metabolism. Direct evidence of the composite nature of these products is obtained in the case of pea-pod cytopentan.

The cytopentan when first prepared was shown to be nitrogen-free by Kjeldahl's method, contained 28 % of ash (abnormally high) and gave a pentose value (on the ash-free weight) = 78 % approx.

On dissolving this in caustic soda, with a view to reprecipitation and

purification as described, the soda was inadvertently over-neutralised, and it was found that the acetic acid had precipitated most of the product. This on drying gave an ash value of 10·6 % and a pentose value of 85 % (ash-free). The remaining liquid gave a very small quantity of a white solid when treated with alcohol, and this product contained 4·4 % ash and 43 % pentose. The majority of the pentans, however, were not precipitated from alkaline solution by the addition of excess of acetic acid; highly opalescent solutions only were obtained, and flocculation did not take place until after addition of alcohol.

The Pentose Estimations on Cytopentans.

Source of cytopentans		Wt. taken	Wt. of 1st precipitate g.	Wt. of soluble portion g.	Wt. of insoluble portion g.	Soluble %	Insoluble %	Ash %	Pentose (on ash-free product) %
Onions	(i)	0·1198	0·0450	0·0041	0·0409	3·45	38·75	2·5	39·75
	(ii)	0·1277	0·0472	0·0032	0·0440	3·51	38·81	2·5	39·80
	(iii)	0·1191	0·0424	0·0037	0·0394	2·28	37·97	2·5	38·94
Turnips	(i)	0·1808	0·1374	0·0046	0·1328	2·56	76·87	5·9	81·70
	(ii)	0·2011	0·1466	0·0049	0·1417	2·46	73·57	5·9	78·18
	(iii)	0·1303	0·0959	0·0040	0·0919	0·72	77·37	5·9	79·76
Oranges	(i)	0·1218	0·0618	0·0029	0·0589	2·41	53·01	4·4	55·45
	(ii)	0·1529	0·0813	0·0046	0·0767	3·03	53·96	4·4	56·44
Apples	(i)	0·1227	0·0463	0·0028	0·0435	2·31	39·96	3·1	41·25
	(ii)	0·1311	0·0484	0·0038	0·0446	3·19	38·25	3·1	39·48
Cabbage	(i)	0·1832	0·0899	0·0051	0·0848	2·81	49·46	14·3	57·73
	(ii)	0·1597	0·0750	0·0053	0·0697	3·34	47·24	14·3	55·13
Pea-pod	(i)	0·1186	0·0664	0·0046	0·0618	3·90	56·90	28·7	79·80
	(ii)	0·1900	0·1063	0·0069	0·0994	3·49	56·60	28·7	77·99
(total cytopentans)									
A. Pea-pod	(i)	0·1495	0·0658	0·0071	0·0587	4·79	43·84	4·4	45·02
	(ii)	0·1201	0·0494	0·0072	0·0422	6·04	39·76	4·4	41·59
(alcohol precipitate fraction)									
B. Pea-pod	(i)	0·1898	0·1467	0·0082	0·1385	4·38	76·23	10·6	85·29
	(ii)	0·1838	0·1389	0·0061	0·1328	3·34	75·62	10·6	84·59
(acetic acid precipitate)									

In one or two cases results are slightly low owing to persistent frothing during the distillation of the furfural. The calculations in the last column represent furfural-yielding radicals calculated as pentose.

In the case of the pea-pod cytopentan, the figures under (A) are for the fraction precipitated by alcohol and under (B) for the fraction precipitated by acetic acid.

D. *Preparation and Properties of Cytopectic Acid.*

After the residues had been washed very thoroughly to free them from the last traces of caustic soda, as much water as possible was expressed and then the material was placed in a bath containing 0·5 % ammonium oxalate in water brought just to boiling point. For the 600 g. of turnip residues, the first extraction bath contained 6000 cc. of solution. The bath was allowed to cool slowly and when sufficiently cool the mass was filtered through muslin

on a large Buchner funnel. The extraction was repeated and the two extracts combined. In order to recover any small fibres that might have worked through the muslin, the liquid extract was filtered through glass wool or paper pulp, which clarified it considerably. Concentrated hydrochloric acid was then cautiously added with constant stirring and when only a little acid had been added, a white gel came down in almost solid condition. As the stirring was continued the whole mass became semi-solid owing to the very bulky nature of the gel.

The residues were extracted five times altogether, using decreasing quantities of ammonium oxalate solution, and the extracts were precipitated by acid and the precipitates combined. The whole of the gel was then filtered through paper in a wide funnel and pressed in large Buchner funnels. The essential point in the preparation of the dry material is the elimination of the excess of hydrochloric acid which is held very tenaciously by the colloid. It was found best to suspend the substance in muslin bags in large quantities of distilled water, and to express the liquid frequently from the gel. This must not be repeated very often however, as the gel tends to take up still larger quantities of water and becomes difficult to press on the filter, clogging the pores of the paper immediately. Most of the acid is eliminated in this way, by water, and the remainder comes out in washings of 50 % alcohol. Finally the mass was placed in graded strengths of alcohol as described for the cytopentans, and lastly in ether, when it was dried off as a white flaky solid.

The cytopectic acid obtained in this way was further purified before carrying out estimations.

The cytopectic acid from cabbages was found to contain 0·6 % nitrogen and was digested with pepsin in dilute HCl. After washing and drying by the method described above, the nitrogen content was reduced to 0·2–0·3 %, corresponding with a protein content of less than 2 %.

For analysis, the cytopectic acid prepared by the above method was subjected to a further purification, which in certain cases was repeated. It was dissolved in about 50 times its weight of $N/2$ ammonia solution; complete solution required about 2 hours. The syrupy liquid was then placed in a tall cylinder and a rapid current of air passed through a scrubber protecting the solution from dust, etc. The object of this was the elimination of the excess of ammonia, thus preventing the formation of a large quantity of ammonium chloride on precipitating the gel with hydrochloric acid. Thus the minimum of acid could be used. The current of air was continued for several hours, and then the solution filtered through two thicknesses of filter paper on a small Buchner funnel.

A small quantity of hydrochloric acid was added and the solution stirred; after some time the solution was almost a solid gel of cytopectic acid. This was washed with water, pressed on a Buchner funnel and allowed to stand in several changes of water. This must not be left more than a day or two, as the substance tends to form a very syrupy gel which is difficult to filter. This

was avoided by allowing to stand in changes of 50 % alcohol and filtering and pressing between each change. This washing treatment was continued until silver nitrate gave only the slightest turbidity with the filtrate. The product was then allowed to stand in gradually increasing strengths of alcohol, 60–75–80–95 %, and finally in absolute alcohol. This was then washed out with ether and the product was air-dried in a current of air from a fan. The final drying of the product for estimation was carried out over P_2O_5 in vacuo at 100° and frequently took from 3–4 days.

The Pentose Estimations on Cytopectic Acid.

The pure product was dried to constant weight at 100° in vacuo over phosphorus pentoxide and the pentose content was determined by the method previously described for the cytopentans.

The values approximate between 40 and 42 %, which limits fairly represent the accuracy of the estimation and indicate the probability of the definite chemical nature of cytopectic acid.

Source		Weight g.	Yield of 1st precipitate g.	Wt. of soluble portion g.	Wt. of insoluble portion g.	Soluble portion %	Pentose (on ash-free product) %
Pea-pod	(i)	0·2628	0·1081	0·0067	0·1014	2·49	40·93
	(ii)	0·3387	0·1347	0·0066	0·1281	1·96	39·65
Turnip	(i)	0·1830	0·0775	0·0062	0·0713	2·86	42·10
	(ii)	0·1859	0·0777	0·0053	0·0724	3·41	42·05
Onion	(i)	0·0990	0·0364	0·0011	0·0353	0·15	41·21
	(ii)	0·1872	0·0716	0·0032	0·0684	1·93	39·60
Orange	(i)	0·1710	0·0677	0·0016	0·0661	0·52	42·00
	(ii)	0·1849	0·0744	0·0033	0·0711	1·79	41·57
Cabbage	(i)	0·0968	0·0359	0·0024	0·0335	2·50	40·26*
	(ii)	0·1079	0·0397	0·0034	0·0363	3·17	38·73*
Apple	(i)	0·1447	0·0587	0·0027	0·0560	1·87	42·61
	(ii)	0·1786	0·0711	0·0024	0·0687	1·35	41·67

* Contains 0·3 % nitrogen.

Analysis of Cytopectic Acid.

The modified furnace due to Dennstedt was used for all the combustions.

The results (Table, p. 653) are calculated on the ash-free substance and it will be seen that each analysis differs very little from the average of the whole, except in the case of the orange cytopectic acid, which gives a lower carbon content and corresponding increase in oxygen percentage.

The Optical Rotation of Cytopectic Acid.

In order to obtain a further indication of the purity of the preparations, the values for the specific rotation for each product were determined.

Solutions in half-normal ammonia were prepared, but owing to the very viscous and, in some cases, cloudy solutions obtained the use of a very small concentration was unavoidable. Hence, a very slight error in the readings

Source		Carbon %	Hydrogen %	Oxygen %	Ash %
Turnips	(i)	41·81	5·86	52·33	0·15
	(ii)	42·08	5·57	52·37	0·15
Average		41·95	5·71	52·34	0·15
Onions	(i)	42·59	5·71	51·70	0·24
	(ii)	43·15	5·59	51·26	0·24
	(iii)	42·91	5·60	51·49	0·24
Average		42·88	5·63	51·49	0·24
Pea-pods	(i)	42·76	5·20	52·04	0·78
	(ii)	42·37	5·61	52·02	0·78
	(iii)	42·62	5·11	52·27	0·78
Average		42·58	5·31	52·11	0·78
Apples		42·41	5·70	51·89	0·85
Cabbages*	(i)	41·76	5·33	52·91	0·48
	(ii)	41·89	5·34	52·77	0·48
Average		41·82	5·34	52·84	0·48
Average for the above products		42·33	5·54	52·13	—
Orange	(i)	41·22	5·43	53·35	0·33
	(ii)	41·30	5·59	53·11	0·33
	(iii)	41·13	5·53	53·34	0·33
Average		41·22	5·52	53·26	0·33

* The cabbage cytopectic acid contained also 0·3 % nitrogen.

was considerably magnified in the final result. However, even in the case of the pea-pod and onion products, where solutions of only 0·13 % were used, results within 5 % of each other were obtained and all the values obtained are within the rough limits $[\alpha]_D^{20} = +260°$ to $+280°$. There was no doubt that the orange product gave a slightly lower value, as in this case 1·0 % solutions were obtainable, and two different solutions gave very definite readings and a final result $[\alpha]_D^{20} = +240°$.

Source		G. per 100 cc.	Average rotation	Specific rotation $[\alpha]_D^{20}$
Turnip		0·4741	+1°·28	+272°
Pea-pod		0·1327	+0°·35	+263°
Onion*		0·1463	+0°·41	+280°
Cabbage		0·3882	+1°·02	+263°
Apple		0·1336	+0°·38	+284°
Onion†		0·4478	+1°·17	+262°
Orange	(i)	1·018	+2°·45	+240°
	(ii)	1·1206	+2°·68	+239°

* Purified from ammonia solution.

† Purified from caustic soda solution.

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LXXIX. THE QUANTITATIVE ESTIMATION OF THE FAT-SOLUBLE FACTOR.

By SYLVESTER SOLOMON ZILVA and MASATARO MIURA.

From the Biochemical Department, Lister Institute.

(*Received September 28th, 1921.*)

The technique hitherto employed in the estimation of the fat-soluble factor has been of a more or less qualitative nature. The substance to be tested has usually been incorporated in a basal diet free from the factor and the potency determined by the tendency of the animal to grow. As the work on the fat-soluble factor progressed it became evident that a quantitative method was desirable for the estimation of the principle in natural products as well as in various fractions obtained in chemical manipulations. No chemical method for establishing the presence of the accessory factors is at present available and we therefore had recourse to the study of suitable conditions under which results of a quantitative nature could be obtained with the biological method now in use.

In the case of the antiscorbutic and the antineuritic factors, quantitative methods have been employed in this Institute for some time. The technique used in the estimation of these factors lends itself more to quantitative work, since the animals employed for the purpose, namely guinea pigs, monkeys, and pigeons can be fed by hand without difficulty, thus affording the opportunity of administering definite quantities of the tested material. Moreover, since the dietetic deficiency in these cases manifests itself by symptoms of well-defined diseases such as scurvy and polyneuritis, the minimum dose necessary to prevent the onset of the disease can be determined and used as a standard of comparison. The deficiency of the fat-soluble factor in the diet of rats, however, does not manifest itself by clinical symptoms with such regularity as could be utilised for comparative purposes. It is well known that the occurrence of keratomalacia in rats subsisting on a fat-soluble-free diet is irregular and consequently it cannot be employed as a standard. We were, therefore, obliged to restrict ourselves to observations on the growth. This, although somewhat vitiated by individual variations, has nevertheless the advantage of being a regular manifestation; rats which have ceased to grow owing to a deficiency of the fat-soluble factor seldom fail to respond to the addition of the active principle.

We do not intend to describe all the numerous experiments which were performed in connection with this inquiry and shall only discuss the conditions

under which we find that quantitative results can be obtained in the estimation of the fat-soluble factor.

At the outset it is essential to point out that a healthy stock of rats must be employed in order to ensure good results. It is evident that any restriction in growth produced by factors other than the deficiency of the fat-soluble factor will lead to erroneous conclusions. Animals weighing 50–60 g. are placed on the usual basal diet, and, if the diet is properly purified, the majority of them show only little growth during the first 10–15 days after which time they cease to grow altogether. Some vigorous rats in spite of the deficiency grow to a marked extent during the first few weeks. Such animals which form only a small percentage of the total number employed are useless for quantitative work, although they can be employed for the ordinary detection tests of the fat-soluble factor. The animals are kept for three to four weeks on the basal diet before the actual testing commences. By the end of this period the rats have not been gaining in weight for some time and any addition of the factor produces a definite response in increase of weight. No rats which have at this stage attained a weight higher than 70 g. are suitable for testing purposes.

In estimating the potency of a substance it is administered in known quantities separately without being incorporated in the diet, the mode of administration varying with the character of the substance to be tested. Our work was confined mainly to oils which are liquid at ordinary temperature such as fish oils, and fats which are solid at ordinary temperature such as butter, margarine, etc.

In the case of liquid oils the following procedure is adopted. The oil is delivered in drops of known weight from a pipette into a small pellet of the basal diet containing a depression and is then covered with a little more of the powdered diet. These pellets are given with the aid of a spatula to the animals which have ceased growing. The rats soon become used to take the dose without difficulty and consume it at once. The daily dose of the basal ration can then be served out. The desired quantity of oil can also be delivered directly into the mouth of the animal. This procedure although quicker requires a certain skill and is unsuitable for routine work when delegated to laboratory assistants. In the case of very potent oils such as cod liver oil, when less than a drop is required, the oil is previously diluted to the desired strength with an inactive oil such as oxidised olive oil and a drop of the diluted oil is employed.

Solid fats are previously melted at a low temperature and the requisite number of drops of known weight are allowed to solidify and are fed with a spatula before the animals receive the basal diet. This procedure is decidedly quicker than weighing each dose separately.

As a standard of comparison we take the least dose which induces definite growth after the animals have ceased to grow owing to the deficient basal diet. This of course has the disadvantage of being a relative standard which might vary with the individual observer, but our experience leads us to believe that it is the only reliable standard under the circumstances and that it is

possible to obtain fairly accurate results with it. Our original intention was to take the minimum dose required to produce normal growth but it was found that the magnitude of such a dose could not be sharply defined from the weight curves. When the dose was gradually reduced a point was reached at

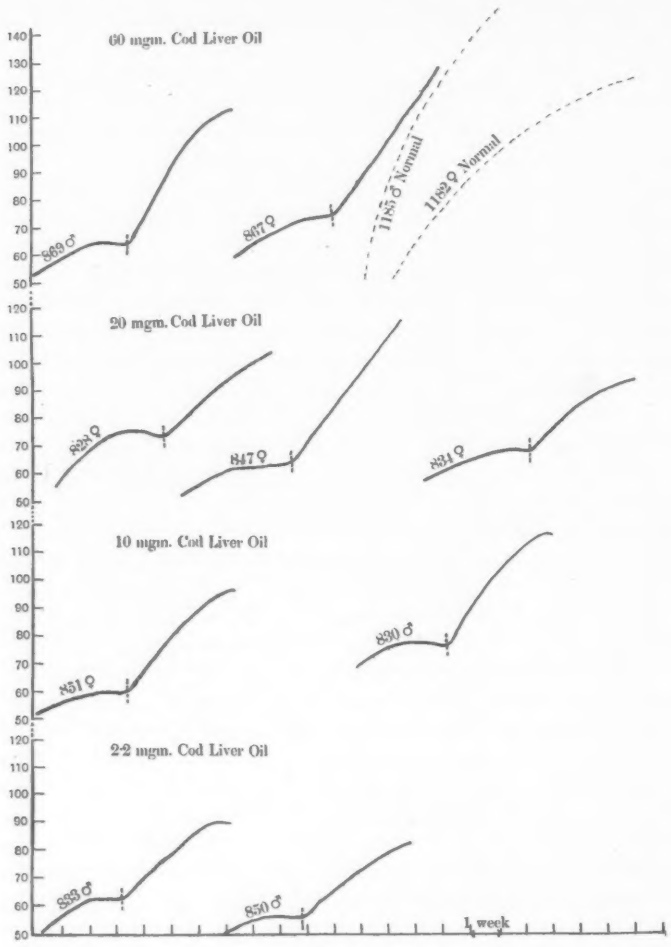

Fig. 1.

which definite growth was still obtained, but any further reduction made the dose inadequate to promote appreciable growth in the majority of the animals. This is illustrated by Figs. 1 and 2 which represent the growth curves of rats which received doses of various magnitudes of a very potent cod-liver oil.

Normal growth was obtained with about 20 mgms. of the oil and the diminution in the rate of growth brought about by reducing the dose was gradual. With 1·7 mgm., *i.e.* 1/12 of a drop (obtained by diluting the original oil), definite growth was still obtained. On reducing the dose to 1·4 mgm. no appreciable growth was recorded. The line of demarcation at this stage is well marked. We therefore take the minimum dose which induces definite growth for four weeks as our standard. Quite consistent results can thus be obtained and the relative potency of various active substances can be established with fair accuracy. This method has also been utilised lately in another

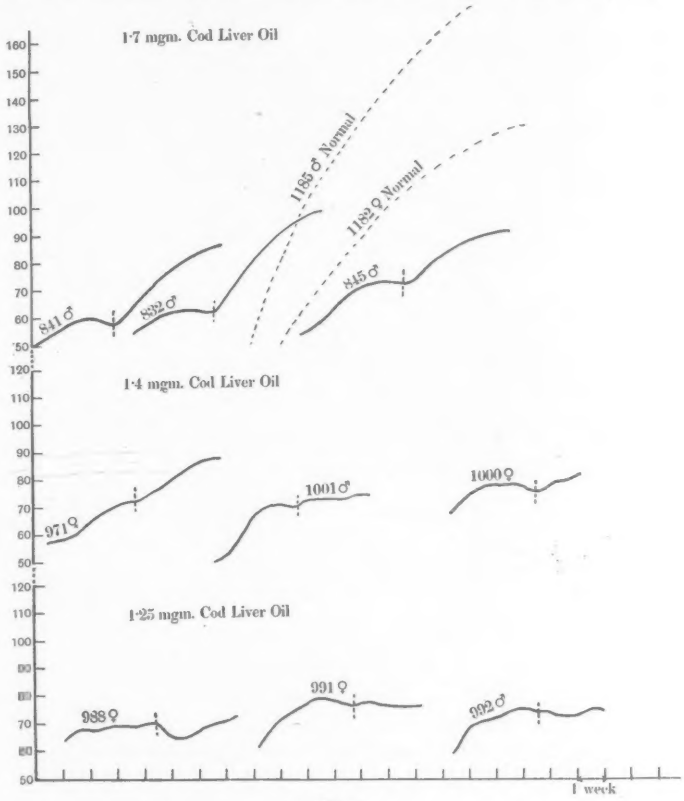

Fig. 2.

laboratory in connection with a joint investigation and equally consistent results were obtained there independently.

In the course of this investigation we had the opportunity of observing the exceptional potency of cod-liver oil. We examined various oils and found that the minimum dose varied from 1·7–5 mgms. The butters which were tested,

on the other hand, were found to be in comparison very much less potent. As will be seen from Fig. 3, the weight curves of animals fed on one of our samples of butter, the minimum dose lies between 200 and 400 mgms. Some butters were even less potent. We [1921] have already expressed the opinion elsewhere that the striking therapeutic value of cod-liver oil is no doubt due to this high vitamin content. In this connection it is interesting to point out that the superiority of cod-liver oil over butter in the treatment of rickets

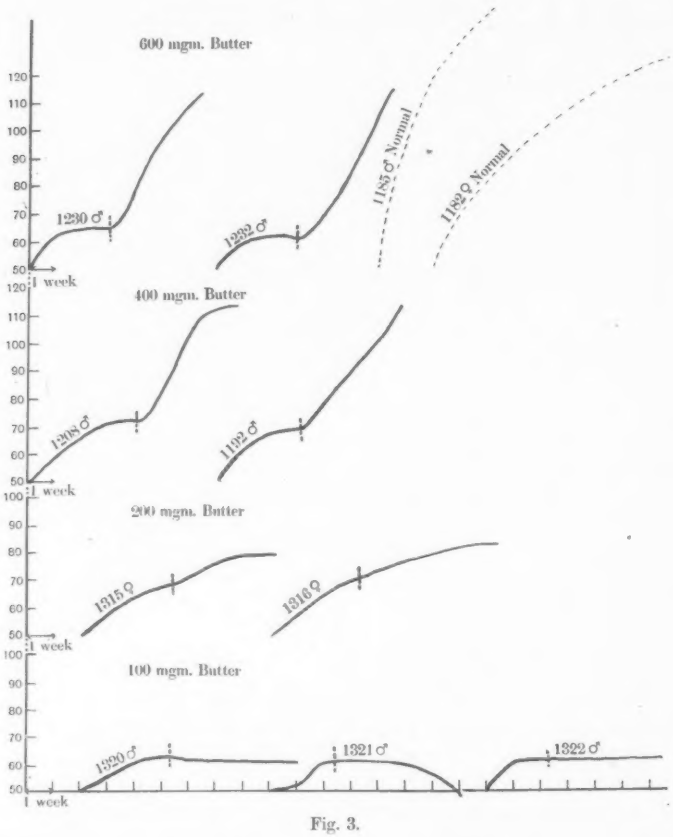

Fig. 3.

was not unknown to some clinicians. Trousseau [1872] in his lecture on rickets, discussing the value of cod-liver oil in the treatment of the disease, refers to butter as follows: "there is one substitute (for cod-liver oil), *butter*, within the reach of all, from which excellent results are obtained; but to obtain such results it is necessary that a large quantity, sixty grams, at least, be taken in the 24 hours." We consider that the difference in the activity of the two

substances is due not to the presence of two different principles as has recently been suggested but to the higher content of the fat-soluble factor in one of them.

The expenses of this research were defrayed from a grant made by the Medical Research Council, to whom our thanks are due.

Summary.

A quantitative method for the estimation of the fat-soluble factor is described.

The exceptionally high potency of cod-liver oil is demonstrated.

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LXXX. DAY AND NIGHT URINE DURING COMPLETE REST, LABORATORY ROUTINE, LIGHT MUSCULAR WORK AND OXYGEN ADMINISTRATION.

By JAMES ARGYLL CAMPBELL and THOMAS ARTHUR WEBSTER.

From the Department of Applied Physiology, National Institute for Medical Research, Hampstead.

(*Received October 26th, 1921.*)

Introduction.

By employing the same subject under varying daily routines it was thought that some information might be obtained regarding the problems of nitrogen metabolism and urinary excretion, if close attention were directed to the composition of the day and night urine. The present results were obtained during three 5-day periods: (i) complete rest in bed, (ii) 6½ hours' ordinary laboratory routine, (iii) 1½ hours' ordinary laboratory routine plus 5 hours' work—13,500 kgm. per hr.—on Schuster's [1921] modification of Martin's bicycle ergometer. For brevity these periods will be called "rest routine," "ordinary routine," and "ergometer routine" respectively. During the "ergometer routine" the subject may be considered to have carried out half-a-day's work of a labourer.

The subject, 28 years of age, 161·2 cm. in height, and 52·4 kgm. in weight, was a skilled laboratory assistant, and accustomed to this type of research. During six months of observation his total nitrogen excretion averaged 9 g. His usual diet was taken, but was controlled mainly to exclude articles which are known to influence greatly the composition of the urine. The average daily diet for each of the 5-day periods was similar in substance. Breakfast was taken about 7.30 a.m., dinner at 1 p.m., light tea at 4 p.m., and supper at 7.30 p.m.

Well-known standard methods were used for the analyses and the average results are given in the appended table.

Composition of average day and night urines under "rest routine," "ordinary routine" and "ergometer routine."

The urine was collected in two samples, day urine between 7 a.m.–5 p.m., night urine between 5 p.m.–7 a.m. in most cases. On a few occasions the day urine included the hours 7 a.m.–10 p.m. and the night urine 10 p.m.–7 a.m. with no important effect on the results. We can find very few experiments

of a like nature in the literature. Osterberg and Wolf [1907] carried out a few days' observations using two different diets, and collecting the urine between 11 a.m.–11 p.m. for the day and 11 p.m.–11 a.m. for the night, allowing 3 hours for "lag in excretion," so that they considered the night urine belonged to 8 p.m.–8 a.m. The largest meal in their case was taken at 7 p.m. In our case the largest meal was taken at 1 p.m. In only a few points can their results be compared with ours owing to the different conditions of experiment.

Water and chloride. Much less water and much less chloride were excreted during the night than during the day. Both were reduced in proportion.

Total nitrogen. Under our conditions of experiment and arrangements for meals, we always noted during all routines a much higher excretion of nitrogen per hour during the day than during the night. Lusk [1917, p. 110] records a similar result for a fasting subject. Osterberg and Wolf [1907] found no regularity as regards the day and night nitrogen under their conditions.

Average hourly day and night results.

	"Rest routine"		"Ordinary routine"		"Ergometer routine"	
	Day	Night	Day	Night	Day	Night
Amount cc.	67·2	31·3	61·2	27·0	58·5	27·6
Acidity %	20·6	70·2	44·8	64·6	50·0	64·0
Titratable acidity (Folin), cc. $N/10$	5·4	18·2	10·4	16·8	11·6	15·4
Total acidity, cc. $N/10$	18·8	36·7	25·4	33·0	28·1	31·6
Total N g.	·386 (100)*	·296 (100)	·428 (100)	·332 (100)	·447 (100)	·357 (100)
Urea N g.	·319 (82·70)	·199 (67·20)	·346 (80·90)	·254 (76·60)	·369 (82·50)	·283 (79·30)
Ammonia N (A) g.	·014 (3·63)	·022 (7·43)	·015 (3·50)	·019 (5·72)	·017 (3·80)	·019 (5·32)
Ammonia N (B) g.	·019	·026	·020	·022	·023	·023
Amino-acid N g.	·005 (1·30)	·004 (1·35)	·005 (1·17)	·003 (0·90)	·006 (1·34)	·004 (1·12)
Creatinine N g.	·032 (8·29)	·029 (9·80)	·032 (7·48)	·031 (9·34)	·030 (6·71)	·028 (7·86)
Uric acid N g.	·008 (2·07)	·005 (1·69)	·007 (1·63)	·005 (1·51)	·007 (1·56)	·005 (1·40)
Undetermined N g.	·007 (2·01)	·035 (12·53)	·023 (5·32)	·018 (5·96)	·018 (4·09)	·018 (5·00)
Chloride (NaCl) g.	·596	·246	·417	·171	·531	·249
Phosphate (P_2O_5) g.	·043	·080	·065	·080	·066	·075
Total S(SO_3) g.	·066 (100)	·066 (100)	·078 (100)	·069 (100)	·073 (100)	·074 (100)
Inorganic S(SO_3) g.	·042 (63·6)	·048 (74·1)	·058 (75·3)	·058 (84·1)	·060 (82·2)	·059 (79·7)
Ethereal S(SO_3) g.	·011 (18·2)	·006 (10·5)	·005 (6·4)	·006 (8·7)	·006 (8·2)	·006 (8·1)
Neutral S(SO_3) g.	·011 (18·2)	·009 (15·4)	·014 (18·2)	·005 (7·2)	·007 (9·5)	·009 (12·1)
Lactic acid per 100 cc.	trace	trace	trace	trace	trace	trace
Calcium (CaO) g.	—	—	·015	·007	·014	·008
Magnesium (MgO) g.	—	—	·007	·006	·006	·007
Purine N g.	·0011	·0017	—	—	—	—

(A) Van Slyke's method. (B) Malfatti's method.
Total acidity is ammonia (B) + titratable acidity.
* Figures in brackets are percentages.

Nitrogen partition. The main difference between the nitrogen partition for the day and for the night under all routines was the greater excretion—both relative and absolute—of ammonia. Other slight differences for all routines were noted; creatinine was excreted in greater amount during the day than at

night; urea, uric acid and amino-acids were excreted in greater amount—usually absolutely and relatively—during the day, than at night. The undetermined nitrogen was excreted in much greater amount at night than during the day under the "rest routine"; under the "ordinary routine" and the "ergometer routine" it was more or less equally distributed, so that it was increased during the day by activity. Osterberg and Wolf [1907] found that it was increased during work. They also found that the ammonia was higher during sleep, and that uric acid was increased during the day. Leathes [1907] previously pointed out that uric acid excretion is most active in the early working hours and very much less at night. Our results, as already shown, support this statement. We also found that under all routines the urea was, relatively and absolutely, excreted in greater amount during the day.

Sulphur excretion. Total sulphur, total sulphates, and inorganic sulphates were excreted in larger quantity per hour per g. nitrogen at night than during the day, because whilst the total nitrogen was decreased at night, the sulphur and sulphates were evenly distributed between the day and night under all routines. This appears to differ from the general idea recorded by Lusk [1917, p. 169] that excretion of sulphur precedes the excretion of nitrogen when a protein containing known quantities of these elements is ingested; but the conditions of our experiments were not quite identical.

The excretion of inorganic sulphate in relatively greater amount at night under all routines indicates probably a special excretion of fixed acid.

For the whole 24 hours, inorganic sulphate was increased whilst ethereal sulphate was decreased by activity.

Phosphate excretion. Phosphates were excreted, under all routines, in greater quantity during the night.

Acidity. Lactic acid was present in traces both during the day and at night under all routines. The acidity per cent. [Leathes, 1919] and titratable acidity were distinctly higher during the night than during the day under all routines, the difference being most marked during the "rest routine," so that activity out-of-bed hastened the excretion of acid.

Purine bodies were excreted in greater amount at night.

Calcium and Magnesium. Calcium was excreted in greater quantity during the day than during the night, whereas magnesium was evenly distributed. Calcium and magnesium obviously followed different courses.

Discussion.

Phosphate tide. Broadhurst and Leathes [1920] and others have shown that there is a phosphate tide, the phosphate being excreted in larger amount during the evening than during the day. Leathes and Broadhurst have endeavoured to discover what is the cause of this tide, and consider that it is independent of the food and not wholly dependent on the acidity. They suggest that it may be due to some special tissue metabolism—nerve or muscle.

It is well known that the curve of phosphate excretion runs more or less parallel to the nitrogen curve but behind it; according to Cathcart [1921] the phosphate or the greater part of it is associated with special nucleoprotein of the food and tissues and may undergo a special form of catabolism. We have obtained some support to this opinion since we found that the purine bodies were excreted in greater amount at night than during the day; at the same time it should be mentioned that we found the uric acid to be excreted in greater amount during the day. We estimated the calcium and magnesium during the "ordinary routine" and "ergometer routine" with the idea that phosphate might show close connection with these substances. However, this was not found to be the case. We could find no connection with muscle or nerve activity in particular, as suggested by Leathes and Broadhurst, since the tide was best marked during the "rest routine."

We observed a close relationship between acidity, ammonia and phosphate excretion. Why is there an increase of acidity at night? It is not sufficient to state that the kidney is more active at night because respiration is depressed. The depression of respiration is no greater than the depression of activity, and the CO_2 excretion by the lungs possibly accounts for the contraction of heart and respiratory muscle which are working at a minimum. Again it will be remembered that the inorganic sulphate was excreted in relatively greater amount at night, indicating some special excretion of acid. It is reasonable to connect this with sleep. One of the well-known theories regarding sleep recorded by Howell [1911] offers an explanation. In this theory sleep is said to be due to accumulation of acid products in cells, these acids being excreted more slowly than they are formed during the day, and thus collecting in the cells with a resulting diminution of activity of the cells, particularly of the brain cortex. We suggest that the cells of the body may not excrete certain fixed acids into the blood until certain amounts are formed in each cell. When this is the case these acids are excreted into the blood and fatigue or sleep is produced. There is presumably a threshold value for excretion, by the cells, of these acids.

Alkaline tide. The so-called alkaline tide is considered here because some text-books state that the urine is more acid at night as there are alkaline tides during the day. We have made numerous observations on our subject during the past six months and find that without exception the urine was more acid just after a meal—probably due to removal of alkaline phosphate in saliva—and less acid about two or three hours after a meal—probably due to removal of HCl in gastric secretion—becoming, in some cases, actually alkaline as estimated by P_H. We find that the alkaline tide was often well marked after the mid-day meal, in more than half the cases being better marked than the tide following breakfast. This is contrary to Leathes' [1919] statement that so-called alkaline tides do not exist except in the morning, and that they are not connected with digestion.

We do not consider that in our experiments the presence of alkaline tide

alone made the urine more alkaline during the day than at night. Two possible tides fell within the day urine and two within the night urine. In some experiments it was arranged that all four meals fell within the day's urine, which thus included all possible tides. This did not increase the acidity at night. One of us [Campbell, 1920] has shown that a tide may follow any meal.

Influence of administration of 35–40 *per cent. oxygen on urinary excretion.*

35–40 per cent. oxygen was administered for 37½ hours spread out over 58 hours' time. We were led to try the effect on urinary excretion, by a case in which oxygen was given in Leonard Hill's [1921] tent to reduce oedema and improve chronic ulcers of the leg. Marked improvement was obtained in the condition of the leg and at the same time the nitrogen, acid and ammonia were doubled in the patient's urine. The cause of this was obscure. It was thought that the absorption of inflammatory products might explain it.

We failed to obtain any marked difference in the urinary composition of our normal subject, the results being much the same under oxygen administration as under ordinary conditions.

Summary.

1. Total nitrogen was decreased at night under all routines specified. Ammonia was increased at night. Creatinine, urea, uric acid and amino-acids were excreted in greater amounts during the day than during the night.

2. Acidity of the urine was distinctly higher during the night under all routines. Activity out-of-bed hastened the excretion of acid.

3. The phosphate tide at night is considered to be due to the increased acidity of the urine. It did not appear to be connected with muscle or nerve metabolism in particular.

4. The sulphur was evenly distributed between day and night.

5. The increase of acidity at night is considered to be due to delayed excretion of certain fixed acids formed in the cells during the day.

6. Administration of 35–40 per cent. of oxygen did not affect the composition of the urine.

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